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Genetic Structure and Demographic History of Two West African Colobine Monkeys:

The Effects of Habitat Fragmentation and Hunting

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RESUMO E PALAVRAS-CHAVE

A destruição e degradação dos ecossistemas naturais representam a principal fonte de declínio da biodiversidade global. Os efeitos isolados e conjugados da fragmentação do habitat por desflorestação e da caça nas regiões húmidas tropicais e subtropicais têm conduzido a reduções drásticas nas densidades populacionais dos primatas. Na África Ocidental tem-se assistido à substituição das práticas agrícolas tradicionais e da caça de subsistência por uma visão económica dos recursos naturais. São muitas as reservas naturais – e até parques nacionais – totalmente transformadas em campos agrícolas que conduziram à extinção das populações de primatas locais. Aliado a isto, a intensidade da caça para consumo e venda comercial de *bushmeat* tem aumentado e são imensas as toneladas de biomassa perdidas anualmente nesta região. Em África, a dependência das populações por *bushmeat* como fonte de proteína faz com que a caça se torne a principal causa de extinção das populações de primatas na África Central e Ocidental. Sozinha, a caça traz consequências devastadoras para a fauna local, mas quando combinada com a fragmentação do habitat por desflorestação, a pressão é exacerbada.

Os primatas arborícolas são um dos grupos mais afetados, estando vários classificados como espécies ameaçadas ou criticamente ameaçadas. Isolados pela fragmentação das florestas, a remoção da sua capacidade de dispersão torna-os presas fáceis para os caçadores. A redução do tamanho populacional efetivo traz consequências ao nível do património genético das espécies, nomeadamente pela perda de diversidade genética. A força da deriva genética é especialmente elevada perante tamanhos populacionais reduzidos, uma vez que as variações nas frequências alélicas se tornam mais bruscas e a probabilidade de fixação de alelos deletérios aumenta. Assim, eventos demográficos como os *bottlenecks* podem ter desfechos drásticos na viabilidade de populações e espécies, podendo mesmo conduzir à sua extinção ao comprometerem a capacidade das espécies se adaptarem a alterações ambientais.

Deste modo, o primeiro objectivo do nosso estudo foi avaliar diversidade genética e história demográfica de duas espécies de cólobos do Parque Nacional de Taï na Costa do Marfim: *Piliocolobus badius* (cólobo-vermelho) e *Colobus polykomos* (cólobo preto-e-branco). Sendo espécies biologicamente e ecologicamente dependentes da floresta, são importantes e interessantes modelos de estudo. O Parque Nacional de Taï (TNP), classificado como Património Mundial pela UNESCO, representa o maior e melhor preservado bloco de floresta tropical húmida da África Ocidental, tendo assim um enorme potencial para suportar populações saudáveis e de elevado tamanho efectivo. Porém, o parque e as suas populações encontram-se isolados devido à desflorestação das áreas circundantes. A caça, apesar de ilegal, é uma presença constante e as espécies de cólobos são sobreexploradas a taxas que superam os 150% da sua capacidade de recuperação. Assim, o Parque Nacional de Taï pode ser considerado como um local apropriado para avaliar os efeitos isolados da caça.

O Parque Nacional de Cantanhez (CNP) na Guiné-Bissau está a ser devastado pela desflorestação e os habitats de floresta encontram-se bastante fragmentados. Novamente, a caça ilegal exerce uma enorme influência sobre os primatas locais e, aliada à fragmentação do habitat, está muito perto de levar à extinção as maiores populações de *Piliocolobus temminckii* e de *C. polykomos* do país, que habitam em Cantanhez. Em 2013 e 2016, os estudos de Minhós *et al*. denunciaram o empobrecimento do património genético destas espécies devido a um *bottleneck* demográfico recentemente provocado pelas actividades antropogénicas. Assim, o segundo objectivo passou pela comparação das populações de

cólobos do TNP e de dados publicados do CNP. Aliado a este objectivo está o terceiro: a quantificação dos efeitos combinados da fragmentação de habitat e caça na remoção de variabilidade genética e potencial evolutivo e viabilidade a longo prazo de populações cruciais de cólobos, que se encontram ameaçadas de extinção nos países considerados e no mundo.

Incidindo nas áreas de genética da conservação e de *fragmentation genetics*, foram inicialmente amplificadas amostras de ADN de *P. badius* e *C. polykomos* do Parque Nacional de Taï, seguido da utilização de diversos *softwares* para aferir a qualidade dos procedimentos e dos dados. O passo seguinte foi a utilização de *softwares* para avaliar (1) a existência de dispersão desigual entre os sexos, (2) diferentes parâmetros de diversidade genética, (3) estrutura populacional e (4) história demográfica.

A nossa abordagem não foi capaz de detectar a existência de dispersão significativamente desigual entre os sexos. Porém, demonstraram uma tendência para que as fêmeas sejam o sexo filopátrico e os machos responsáveis pela dispersão em ambos as espécies. Os resultados contrariam estudos prévios sobre *P. badius* e os motivos podem ser quer metodológicos, quer inerentes à própria ecologia da espécie. Entre todos os primatas, os colóbos possuem um dos mais complexos e variáveis sistemas sociais. A plasticidade comportamental é uma característica inerente das espécies de cólobos e pode ser a resposta para muitas questões.

No que toca à diversidade genética, além da interpretação dos resultados obtidos, realizámos uma pequena revisão bibliográfica de forma a podermos compará-los com outras populações e espécies de colobíneos africanos e asiáticos. A nossa análise confirmou a esperada boa condição genética das populações e, além disso, demonstrou que possuem uma das maiores diversidades genéticas já estudadas, mesmo considerando o número de amostras como factor limitante. Por exemplo, a nossa estimativa da riqueza alélica demonstrou que os valores não são só altos, como ainda existe potencial para que sejam maiores.

Esperávamos encontrar em Taï um único cluster genético, ou seja, a classificação de todos os indivíduos amostrados numa única população: foi exactamente este o resultado obtido para ambas as espécies. Na ausência de barreiras directas à dispersão, de acordo com o nosso conhecimento, não seria de esperar a presença de uma estrutura populacional mais elaborada visto que algo dificilmente o justificaria. Além disto, a pressão da caça é maior nas zonas periféricas do parque o que poderá promover o movimento para o centro e uma maior sobreposição das áreas dos diferentes grupos sociais, facilitando o fluxo genético. A ausência de referenciação geográfica das nossas amostras não permitiu um maior desenvolvimento da questão, pelo que uma abordagem ao nível da paisagem (*landscape genetics*) poderia trazer mais benefícios neste âmbito.

Relativamente à história demográfica, os três *softwares* utilizados não corroboraram os resultados entre si. Foram detectados sinais de estabilidade do efectivo populacional ao longo do tempo, facilitados pelas condições e localização do habitat, que terão permitido a manutenção de elevados tamanhos populacionais ao longo de grandes períodos de tempo estará por detrás dos elevados índices de diversidade genética encontrados. Porém, este cenário não parece perdurar por muito mais tempo, pois foram igualmente detectados sinais de declínio populacional nos colobíneos do TNP, muito provavelmente, devido à pressão exercida pela caça.

A comparação entre o TNP e o CNP sugere que os efeitos conjugados de fragmentação de habitat por desflorestação e da caça impulsionam o declínio a nível genético e de efectivo populacional das populações. A detecção dos sinais de *bottleneck* recentes em Taï resulta, muito provavelmente dos efeitos da caça e das actuais taxas de sobreexploração das espécies de colobíneos. No entanto, os resultados encontrados ao nível do efectivo populacional ainda não se terão traduzido na diversidade

genética das populações. Este facto pode, porventura, ser interpretado pela vantagem dada pela ausência de fragmentação na manutenção do efectivo populacional e assim, de uma elevada diversidade genética.

Por fim, esperamos que os nossos resultados realçem a importância de, no mínimo, reter os presentes efectivos populacionais de *P. badius*, *P. temmicnkii* e *C. polykomos* nos dois parques a fim de assegurar a sua viabilidade a longo prazo. Para que isto seja possível, é crucial desenvolver projectos de conservação da biodiversidade e restauro dos ecossistemas envolvendo as populações humanas locais e alertando para os benefícios sociais, económicos e ecológicos que poderão advir da protecção do património natural.

PALAVRAS-CHAVE: fragmentação do habitat, desflorestação, caça, genética da conservação, primatas

ABSTRACT AND KEYWORDS

The destruction and degradation of the natural ecosystems represent the main source of decline of the global biodiversity. The isolated and combined effects of habitat fragmentation by deforestation and hunting in tropical and subtropical humid regions have led to drastic reductions in population density of primates. In West Africa, many natural reserves – and even national parks – have been totally converted to crop production, which has led to the local extinction of primate populations. Joining this, the hunting for consumption and commercial business of bushmeat has increased and several tons of biomass are being annually lost in the region. The arboreal primates are one of the most affected groups, with many species are considered as endangered or critically endangered. The reduction of the effective population size has consequences in the species genetic pool, namely due to the loss of genetic diversity. Hence, demographic events like the bottlenecks can have drastic effects on population and species viability, and may even lead to extinction by compromising the species ability to adapt to environmental changes.

Our study aimed to analyse the genetic diversity and demographic history of the populations of two colobine species (*Piliocolobus badius* and *Colobus polykomos*) from Taï National Park (Ivory Coast). We found that each species is grouped in a single population within the park and possess one of the greatest genetic diversity among several colobine populations. However, we were able to detect positive signals of both natural ancestral and ongoing bottleneck in both species. The current decline is probably a result of anthropogenic activities. Recent studies provided insights to the impoverishment of the genetic heritage of Cantanhez National Park's colobine populations due to a recent demographic bottleneck with anthropogenic origins, namely habitat fragmentation and hunting. We undertook a conspecific and congeneric comparison between the parks to establish a framework of fragmented versus non-fragmented habitat where hunting is a common threat. The results highlighted the fragility of Cantanhez's colobines.

Finally, we contextualized our data with the current socio-economic situation of the local populations that depend on the natural resources the parks provide. In order to achieve the conservation and the long-term viability of the focal endangered *Piliocolobus badius*, *Piliocolobus temminckii* e *Colobus polykomos* populations, the local human communities must be engaged in the process. Biodiversity conservation and ecosystem recovery must be conducted along with the people, promoting the awareness and the idea that many social, economic and ecological benefits may arise with the protection of the natural heritage.

KEYWORDS: habitat fragmentation, deforestation, hunting, conservation genetics, primates

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1 INTRODUCTION

1.1 HABITAT FRAGMENTATION AND DEFORESTATION

The destruction and degradation of natural ecosystems represents the major source of decline of the global biodiversity (Pereira *et al*., 2010). The Pleistocene and Holocene have been marked by great changes in both fauna and flora, triggered not only by climatic fluctuations (Quéméré *et al*., 2012; Salmona *et al*., 2012; Doughty *et al*., 2013; Nakazawa and Peterson, 2015), but also by anthropogenic factors (Allen *et al*., 2012; Sharma *et al*., 2012; Radespiel and Bruford, 2014; Ram *et al*., 2015; Harrison *et al*., 2016; Mattucci *et al*., 2016). Despite nature's ability to modify the habitat's limits and biodiversity through a geological timescale, the current global impacts of human activities have created a new scenario where the habitat loss rate has exponentially grown to values superior to 1% per year (Radespiel and Bruford, 2014). The magnitude of these human induced changes has led many authors to define this period as the "Anthropocene" (Lewis and Maslin, 2015).

The destruction and degradation of an habitat begins, typically, with its fragmentation (Figure 1.1; Fahrig, 2003; Haddad *et al*., 2015): the division of a large and continuous habitat area into small fragments, isolated from each other by an anthropogenic derived landscape matrix. The fragmentation effects are severe and are felt in all communities and layers of an ecosystem. Besides reducing the habitat's total available and suitable area, it frequently leads to population declines, demographic isolation, reduced resources accessibility and even to the decrease of the evolutionary potential of a population or species by cutting the likelihood of an answer when faced with large-scale environmental changes, like sudden climate fluctuations (Craul *et al*., 2009; Hoffman and Sgró, 2011; Radespiel and Bruford, 2014; Haddad *et al*., 2015; Minhós *et al*., 2016). All these factors bring extinction closer and closer to populations and species. The magnitude of habitat's fragmentation impacts, among other reasons, depends on the kind of landscape matrix originated and the ecological capacity of the species to accommodate it (Pozo-Montuy *et al*., 2011; da Silva *et al*., 2015).

In the tropical and subtropical humid regions, the habitat fragmentation can be seen, essentially, as deforestation, a huge problem that impacts human health (Reddington *et al*., 2015), climate (Lawrence and Vandecar, 2015; Vasconcelos *et al*., 2015; Almana and Cescatti, 2016), and biodiversity, which has been triggering a crescent awareness of both society and scientific community (du Toit *et al*., 2004).

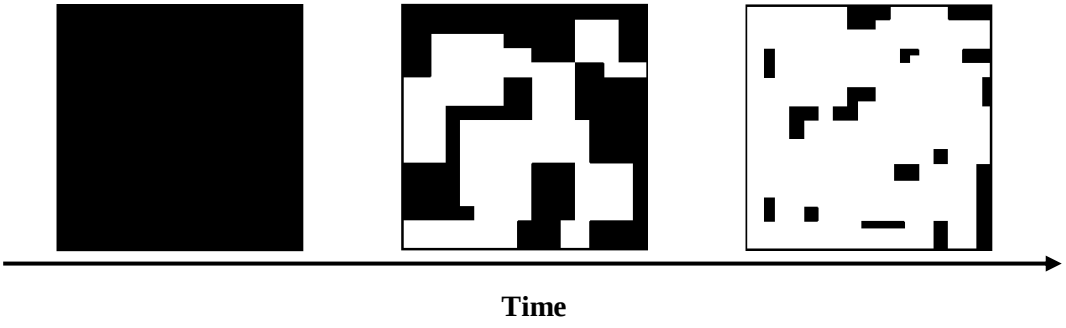


Figure 1.1 – Habitat fragmentation through time. The black color represents the habitat area, and in white the matrix between habitat patches. Adapted from Fahrig, 2003.

Regarding climate, estimations show that deforestation is responsible for 1/4 of global carbon emissions (IPCC, 2014). Recently, the United Nations Framework Convention on Climate Change has emitted new guidelines for the REDD+ programme, aiming to give more momentum to the struggle against carbon dioxide emissions originated by deforestation in underdeveloped countries (UNFCCC, 2016). In Central and South America, intense and extensive logging, agriculture and pastoral practices are responsible for an abrupt deforestation rate (Chatelain *et al*., 2010; Ochoa-Quintero *et al*., 2014; Serio-Silva *et al*., 2015) – that often seeks to respond to national and international requirements –, a scenario totally different from the one present in West Africa. Here the deforestation tends to be a gradual process initiated by selective timber exploitation, followed by charcoal production, traditional shifting agriculture and small-scale crop cultivation (Chatelain *et al*., 2010; Temudo, 2012). However, the traditional shifting agriculture practices have becoming unsustainable as human population density increases (Temudo *et al*., 2015). Despite the contrasting scenarios, they both lead to the loss of biodiversity. Overlying the distribution of the world's tropical and subtropical humid areas to the biodiversity hotspots map (Figure 1.2; Myers *et al*., 2000) – regions with high level of endemism and species richness – it is possible to detect the spatial coincidence between their geographical limits and the regions threatened by deforestation. From India (Ram *et al*., 2015), Indonesia (Goossens *et al*., 2005; Sharma *et al*., 2012), Madagascar (Olivieri *et al*., 2008; Craul *et al*., 2009; Kun-Rodrigues *et al*., 2014), continental Africa (Mbora and Meikle, 2004; Chatelain *et al*., 2010; Chapman *et al*. 2013; Minhós *et al*., 2016; Ruiz-Lopez *et al*., 2016), Central and South America (Pozo-Montuy *et al*., 2011; Benchimol and Peres, 2013; Ochoa-Quintero *et al*., 2014; da Silva *et al*., 2015; Núñez-Regueiro *et al*., 2015; Serio-Silva *et al*., 2015; Sales *et al*., 2016; Cervera and Griffith, 2016; Zanin *et al*., 2016), amid several other places, arrive more and more studies of the negative impacts of habitat's fragmentation by deforestation in the biodiversity. Between 2000 and 2012, 2.3 million km^2 of forest were lost (Figure 1.3; Hansen *et al*., 2013). In these 13 years, the tropical forest area in the world decreased 2101 km^2 per year (Hansen *et al*., 2013). Even though the global deforestation rate is decreasing (Morales-Hidalgo *et al*., 2015), the current rate of 0.08% annual loss recorded between 2010 and 2015 (Keenan *et al*., 2015) is especially relevant for being concentrated on highly valuable ecological areas (Morales-Hidalgo *et al*., 2015). The reasons that lead to deforestation are distinct and are subject of the socio-economic reality of the local populations and countries.

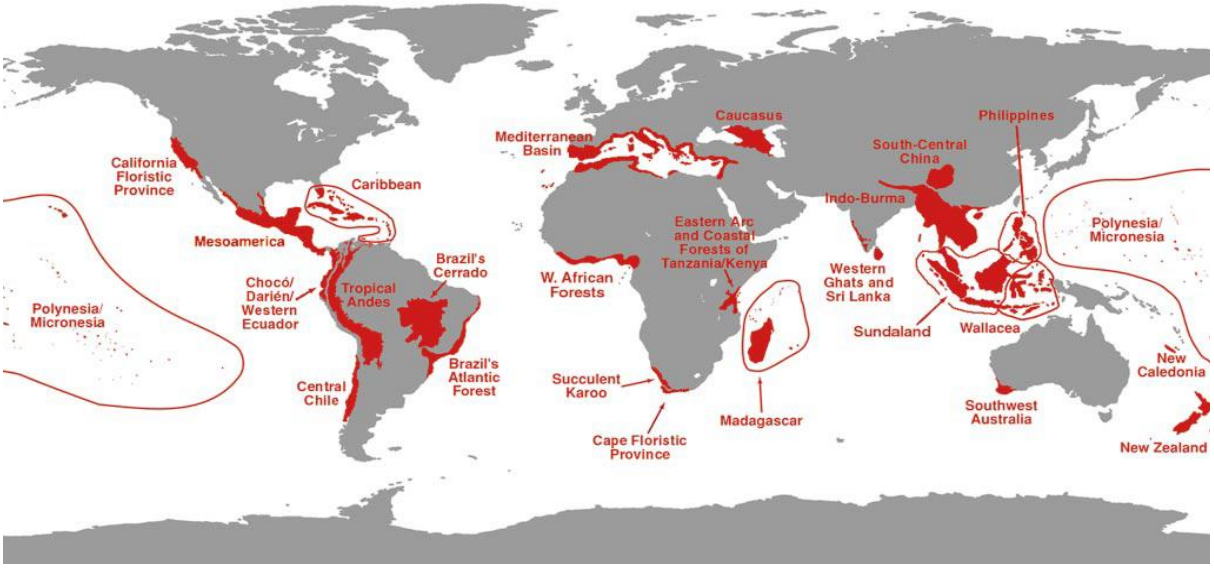


Figure 1.2 – Global biodiversity hotspots. From Myers *et al.* 2000.

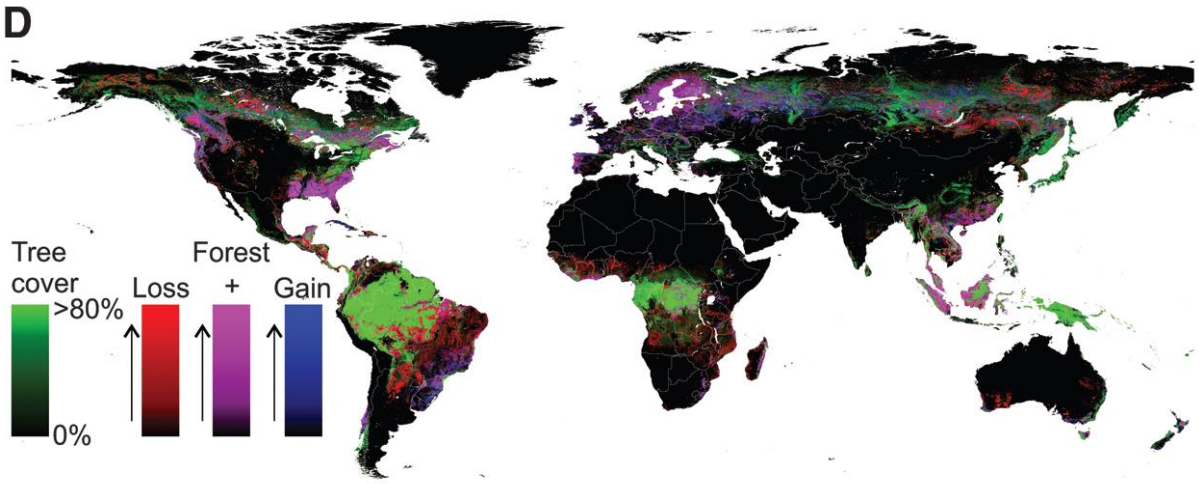


Figure 1.3 – Worldwide tree cover percentage, forest loss and forest gain. From Hansen *et al.* 2013.

1.2 HUNTING FOR BUSHMEAT

In addition to deforestation, hunting for bushmeat also represents an answer to the survival of many human populations that has been jeopardizing biodiversity, mainly in the more impoverished regions of the world. Hunting usually arises from 3 reasons: culture, food and money (Harrison *et al.*, 2016). In underdeveloped countries, hunting was associated to an unavoidable subsistence consumption owing the lack of other protein sources (Cronin *et al.*, 2015). Although, sometimes, this habit was badly interpreted, the absence of alternatives made it justifiable, falling in the concept of "weapons of the weak" created by the political scientist and anthropologist James Scoot in 1985. For humans, bushmeat consumption embodies a risk as well. Zoonosis transmission through the contact amongst humans and infected bushmeat is a reality in Africa (Wolfe *et al.*, 2005). Nonetheless, bushmeat markets have become an important economic segment (Refisch and Koné, 2005a, 2005b; Linder and Oates, 2011; Rovero *et al.*, 2012; Minhós *et al.*, 2013a; Covey and McGraw, 2014; Kun-Rodrigues *et al.*, 2014; Cronin *et al.*, 2015; Harrison *et al.*, 2016). Traditions are falling into forgetfulness as generations go by due to the increasing value given to monetary resources (Costa *et al.*, 2013), in parallel with the arrival of new migrants that carry distinct ethnicities and beliefs (Temudo, 2012). The Southeast Asian tropical region is being totally devastated by hunting, and the forests, even the largest ones, are losing all animal species that weight more than 1 kg (Harrison *et al.*, 2016). The growing ease of access to fire guns and the bushmeat and other animal derived products request has rapidly drained the wild populations in the region. In a tropical forest, a moderate pressure of hunting is enough to disrupt the mammals' community structures (Laurance *et al.*, 2006). The African tropical region is considered one of the highest hunting pressurized regions in the world: estimates show that 1 to 5 million tons of biomass are hunted for bushmeat annually (Laurance *et al.*, 2006). The African populations' food dependency on bushmeat leads hunting to overcome habitat destruction as the main source of primate population havoc in the Central and Western regions of Africa (Figure 1.4; Linder and Oates, 2011; Rovero *et al.*, 2012). By itself, hunting can have devastating consequences for the local fauna, when combined with deforestation, the pressure is exacerbated (Figure 1.5 Benchimol and Peres, 2013; Núñez-Regueiro *et al.*, 2015).

Figure 1.4 – Primate bushmeat. On the left: a *Piliocoblobus temminckii* in Daobly bushmeat market (From Covey and McGraw, 2014). On the right: primate carcasses traded in a market in Guinea-Bissau (From Minhós *et al*., 2013a).

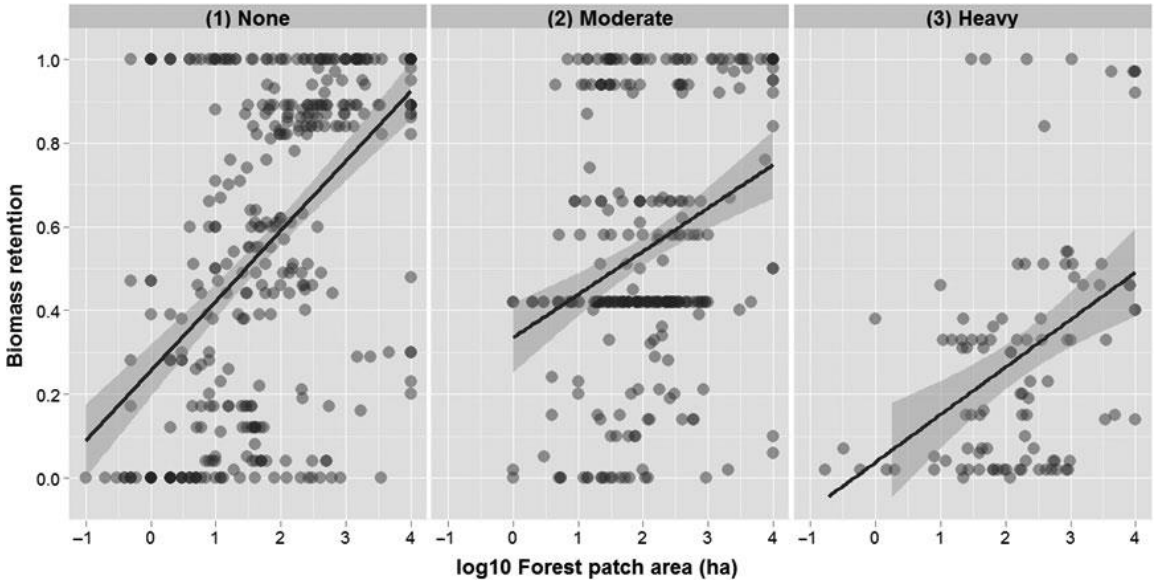


Figure 1.5 – Relationship between forest patch size and total biomass, under different levels of hunting pressure in a Neotropical region. From Benchimol and Peres, 2013.

1.3 PRIMATES' VULNERABILITY

Of all primate taxa, about 60% falls in the IUCN's criteria to be threatened of extinction (IUCN, 2016). Their characteristic forest dependency to find food, shelter and breeding grounds makes them especially fragile towards any kind of change in habitat. The primate species' vulnerability to extinction is quite variable (Isaac and Cowlishaw, 2004): a function of not only the threatening agent, but also their intrinsic biological features, as the generation time, diet, social system and body size. The diurnal forest-dependent primates are easily detected due to the communication noise, a probability that is enhanced by a larger group size (Rovero *et al*., 2012). Adding to these two traits the highly predictable choice of feeding sites, we have the perfect cocktail for the failure of resistance against deforestation and hunting, the two main sources of worldwide decline of primate populations (Rovero *et al*., 2012). If we look at

the latest IUCN Primates in Peril report, listing the currently 25 most endangered primate species or the world, from the smallest – like the dwarf lemurs, galagos and tarsiers – to the biggest – like Grauer's gorilla (*Gorilla beringei graueri* Matschie, 1914) and the Sumatran orangutan (*Pongo abelii* Lesson, 1827) –, the reasons for being listed range between the loss of habitat (mainly through deforestation) and hunting (for bushmeat consumption or to supply the illegal trade market), either isolated or combined (Schwitzer *et al*. 2014). One important fact to have in mind is that primates are also involved in many ecological processes – as seed dispersal for example (Koné *et al*., 2008) –, therefore, the consequences of their disappearance are felt far beyond the population level (Pozo-Montuy *et al*., 2011; Chapman *et al*. 2013; da Silva *et al*., 2015).

1.4 THE COLOBINAE

Given the referred cocktail recipe, perhaps it is not a surprise that the first primate declared extinct in the XX century was *Piliocolobus waldronae* Hayman, 1936, Miss Waldron's red colobus, native to West Africa (Oates *et al*., 2000). Classified as critically endangered by the International Union for Conservation of Nature (IUCN; Oates *et al*., 2016), *P. waldronae* exhibits – or exhibited, since individuals are not seen for almost three decades – all the cocktail elements that classify a primate as highly vulnerable towards deforestation and hunting. This species belongs to the Cercopithecidae family, the Old World monkeys. The family is divided in two subfamilies, *Colobinae* (colobines or leaf-eating monkeys) and *Cercopithecinae* (cercopithecines or cheek-pouched monkeys) based on different morphological and ecological traits. The designation of colobines as leaf-eating monkeys derives from their adaptations to a folivorous diet, including a ruminant-like multi-chambered stomach (Delson, 1975; Ting, 2008). The diet, the availability of food resources and the protein-fibre ratios influence on a large scale the distribution and abundance of colobines (Wasserman & Chapman, 2003; Fashing *et al*., 2007; Gogarten *et al*., 2012; Hanya & Chapman, 2013; McGraw *et al*., 2015). The first fossil found of an African colobine dates of the late Miocene (Kingston *et al*., 2002). Scarce in Africa until the Pliocene, this period was marked by the species radiation, which resulted in the division of the African and the Asian clades (Ting, 2008; Chang *et al*., 2012; Rossie *et al*., 2013). With a large body size and partially adapted to a terrestrial lifestyle, the ancestral taxa were pretty different from the existent. The first colobines morphologically similar to the current ones and, therefore, adapted to an arboreal lifestyle have only emerged during the Pleistocene (Frost and Alemseged, 2007; Ting, 2008). The existent African colobines are grouped in three genera: *Colobus* (black-and-white colobus), *Piliocolobus* (red colobus) and *Procolobus* (green colobus). This great diversity could have been originated by the past climatic oscillations (such as the glaciations) that pushed the populations to find refuge in isolated forest fragmented, promoting their divergence. Upon return of favourable climatic conditions, the individuals could undergo periods of adaptive radiation. In 2008, Nelson Ting demonstrated the relationship between the African colobines through mitochondrial DNA (mtDNA) sequencing (Figure 1.6). The results showed that the red and the olive colobus species are phylogenetically close, and the black-and-white colobus diverged from the others during the African radiation, 7.5 Ma (late Miocene).

The African colobines are particularly affected by habitat fragmentation and hunting since they are forest-dependent arboreal species whose social groups can be considerably large, and present different levels of diet flexibility (Rovero et al., 2012; Minhós et al., 2016).

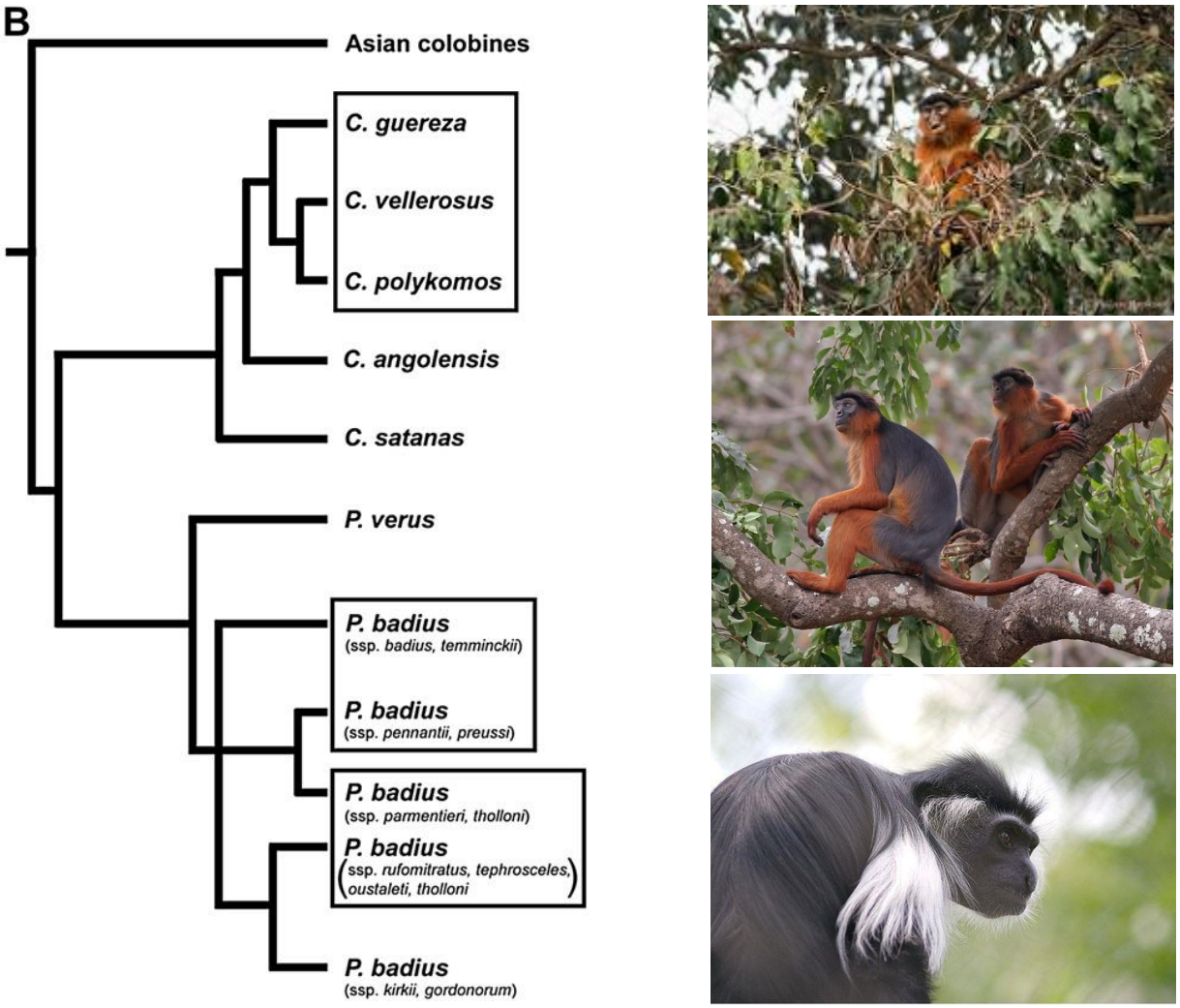


Figure 1.6 – On the left: mitochondrial relationships between the colobine species (From Ting, 2008). On the right: top - *Piliocolobus temminckii* (photographed by Alan Hopkins); middle - *Piliocolobus badius* (photographed by Aat Bender); bottom - *Colobus polykomos* (photographed by Ryan Poplin). The use of these photographs was permitted by the license terms available for each online.

1.5 THE *PILIOCOLOBUS* GENUS – RED COLOBUS

Recently, the unstable taxonomic classification of the red colobus species complex (Oates and Ting, 2015) has been updated due to diversity exhibited in pelage, vocalization and cranial morphology. The former subspecies of *Piliocolobus badius* Kerr, 1792, are now classified as species, in a total of 13 (according to the IUCN): *Piliocolobus badius*; *Piliocolobus waldronae*; *Piliocolobus bouvieri* de Rochebrune, 1887; *Piliocolobus epieni* Grubb and Powell, 1999; *Piliocolobus gordonorum* Matschie, 1900, *Piliocolobus kirkii* Gray, 1868; *Piliocolobus oustaleti* Trouessart, 1906; *Piliocolobus pennantii* Waterhouse, 1838; *Piliocolobus preussi* Matschie, 1900; *Piliocolobus rufomitratus* Peters, 1879; *Piliocolobus temminckii* Kuhl, 1820; *Piliocolobus tephrosceles* Elliot, 1907; and *Piliocolobus tholloni* Milne-Edwards, 1886. However, what has not changed is the classification of the majority of these species as endangered or critically endangered, including two amongst the 25 most endangered primate

species of the world (Schwitzer *et al*., 2015; IUCN, 2016). Their equatorial distribution extends from Gambia to Zanzibar in a discontinuous manner (Figure 1.7, Ting, 2008). The reasons behind this fragmentation are still uncertain and may attributed to natural barriers like rivers, or anthropogenic destruction of the forests in Central Africa (Anthony *et al*. 2014), underlying the fact that they are exclusively arboreal. The already cited phylogenetic study (Ting, 2008) revealed the existence of three main red colobus clades (Figure 1.6): West Africa (*P. badius* e *P. temminckii*), Western Equatorial Region e Congo Basin (*P. pennantii*, *P. preussi* e *P. tholloni*) and Central Africa (*P. tholloni*, *P. rufomitratus*, *P. tephrosceles*; and a sister taxon that groups *P. kirkii* e *P. gordonorum*). It is estimated that the split between the clades occurred 3 Ma. Regarding the ecology, red colobus live in large social groups with an average of 25 to 40 individuals, that may exceed 80 individuals. Generally, the groups contain several males and females (with a minimum of three adult males and, at least, twice the number of adult females), being the first philopatric and the females the dispersing sex (Struhsaker, 2000; Minhós *et al*., 2013b). They have a folivorous diet dependent of protein-fibre ratios, feeding fundamentally on young leaves and fruits (Wasserman and Chapman, 2003; Korstjens and Dunbar, 2007; Gogarten *et al*., 2012; Hanya and Chapman, 2013; McGraw *et al*., 2015). Chimpanzees (*Pan troglodytes verus* Schwarz, 1934) and red colobus establish a predator-prey system between them in several regions of their distribution (Boesch, 1994; Noë and Bshary, 1997). Besides chimpanzees, their natural predators include the crowned eagle (*Stephanoaetus coronatus* Linnaeus, 1766) and the leopard (*Panthera pardus* Linnaeus, 1758) (McGraw and Bshary, 2002). The high predation pressure can give an explanation to the red colobus large social system structure, as well as to their interspecific relationships with other primates of the same family, such as *Cercopithecus diana* Linnaeus, 1758 (Noë and Bshary, 1997; Korstjens *et al*. 2002; Buzzard, 2010) and *Cercocebus atys* Audebert, 1797 (McGraw and Bshary, 2002) in Taï National Park (Ivory Coast), and *Colobus polykomos* Zimmermann, 1780, in Cantanhez National Park (Guinea-Bissau; Gippoliti & Dell'Omo, 1996).

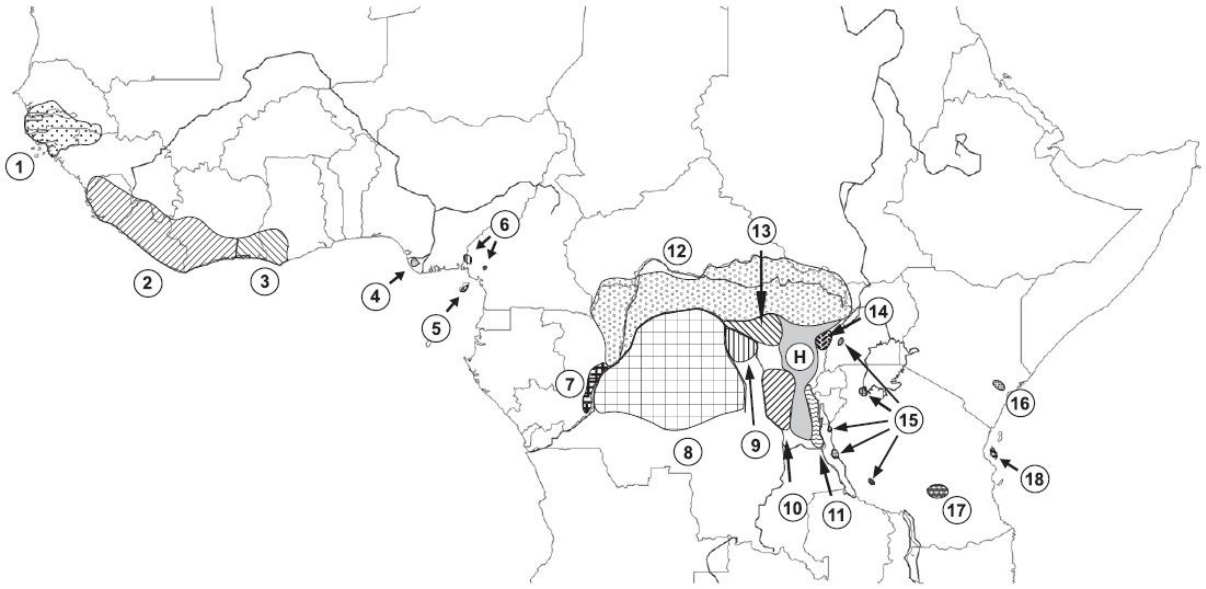


Figure 1.7 – Geographical distribution of the red colobus species according to the previous taxonomic classification (from Ting, 2008). Current species classification: 1-*P. temminckii*, 2-*P. badius*, 3-*P. waldronae*, 4-*P. epieni*, 5-*P. pennantii*, 6-*P. preussi*, 7-*P. bouvieri*, 8-*P. tholloni*, 11-*P. oustaleti*, 15-*P. tephrosceles*, 16-*P. rufomitratus*, 17-*P. gordonorum*, 18-*P. kirkii*.

1.6 THE *COLOBUS* GENUS – BLACK-AND-WHITE COLOBUS

The genus *Colobus* has a more stable taxonomic classification (Figure 1.6) with five recognized arboreal primate species that are nearly continuously distributed along equatorial Africa (Figure 1.8): *Colobus polykomos*; *Colobus angolensis* Sclater, 1860; *Colobus guereza* Rüppel, 1835; *Colobus satanas* Waterhouse, 1838; and *Colobus vellerosus* Saint-Hilaire, 1834. While *C. angolensis* e *C. guereza* have no major concerns regarding their conservation, *C. polykomos*, *C. satanas* and *C. vellerosus* are held as vulnerable species (IUCN, 2016). The pelage is one the traits that distinguishes the species, exhibiting different combinations of black, white and grey on the head, back and tail. The mtDNA phylogenetic analysis revealed that the black-and-white colobus started diverging between the end of the Pliocene and the beginning of the Pleistocene (Ting, 2008). *C. satanas* has begun diverging 3.5 Ma, followed by *C. angolensis* 2.1 Ma, *C. guereza* 1.6 Ma and lastly *C. polykomos* and *C. vellerosus* – considered as a sister taxon – started diverging from each other 200000 years ago. This phylogenetic proximity may be at the origin of the subspecies *Colobus polykomos dollmani* Schwarz, 1927, originated by hybridisation amid *C. polykomos* and *C. vellerosus* in their distribution limits, the Sassandra-Bandama interfluvial region (Ivory Coast) (Gonedelé Bi *et al*., 2006; Gonedelé Bi *et al*., 2012; Gonedelé Bi *et al*., 2014). The black-and-white colobus social groups tend to be formed by less than 20 individuals and, unlike most of the species that multi-male multi-female, in *C. guereza* and *C. polykomos* a polygynous system seems to predominate (Korstjens *et al*., 2002) and both sexes disperse (Korstjens *et al*., 2002; Minhós *et al*., 2013b). In spite of having a diet based on protein-fibre ratios, it is much flexible than the red colobus, feeding on the one hand on young leaves and fruits and, on the other hand on seeds and mature leaves (Wasserman and Chapman, 2003; Fashing *et al*., 2007; Korstjens and Dunbar, 2007; Hanya and Chapman, 2013; Djègo-Djossou *et al*., 2015; McGraw *et al*., 2015).

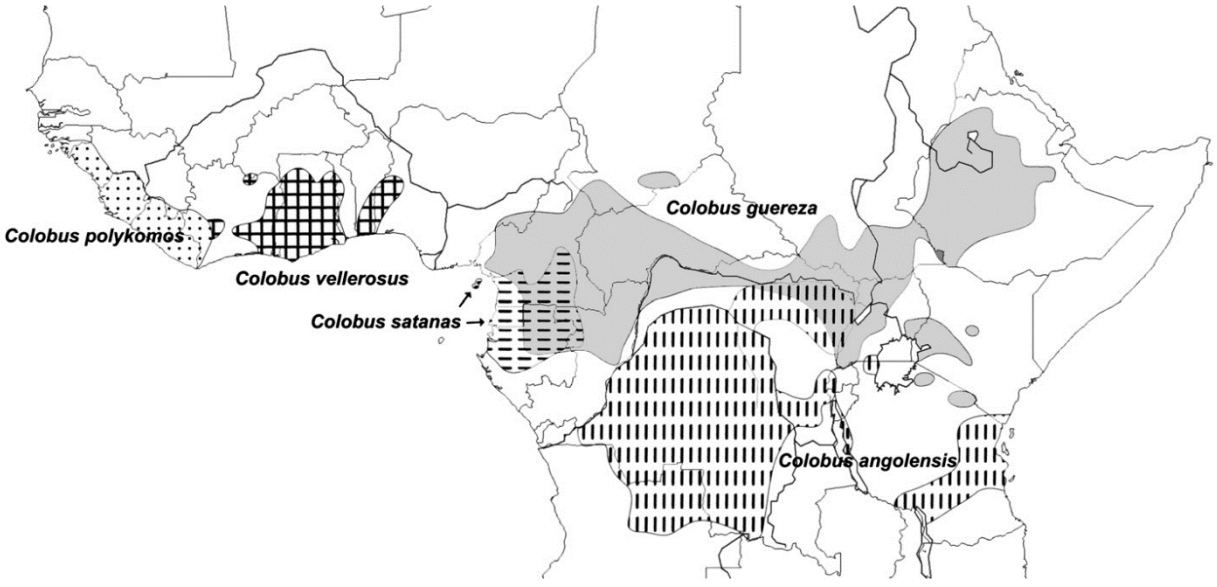


Figure 1.8 – Geographical distribution of the black-and-white colobus species (from Ting, 2008).

1.7 STUDY SPECIES

Piliocolobus temminckii, *Piliocolobus badius* and *Colobus polykomos* (Figure 1.6) represent Africa's westernmost colobine species. These species generally live in sympatry and share a very similar ecology, associated to the arboreal lifestyle (Korstjens *et al*., 2002; Minhós *et al*., 2016). Present in Senegal, Gambia and Guinea-Bissau, with a decreasing population trend, *P. temminckii* are classified as an endangered species by the IUCN (2016). Their social structure is typical of the red colobus (multi-male, multi-female) and the groups tend to have 12 to 65 individuals. The dispersal is mediated by females, being this considered as a patriarchal society. *P. temminckii* are phylogenetically and ecologically analogous to *P. badius* (Ting, 2008). The latter's distribution ranges continuously along the strip of coastal countries stretching from Guinea to Ghana. Hence, the biogeography of these two species commonly coincides with *C. polykomos* distribution. They are social-ecologically different from red colobus: the black-and--white colobus live in smaller social groups (less than 20 individuals), both sexes disperse and they have a more flexible diet (Korstjens and Dunbar, 2007; McGraw *et al*., 2015). This higher flexibility can be seen as an advantage towards the threats that target them. The denoted genera, by sharing the same biogeography, but distinct social-ecological qualities, are particularly interesting as models to evaluate the impact of habitat degradation in arboreal species.

1.8 STUDY SITES

Our study comprises two focal sites in West Africa: the principal represented by Taï National Park in Ivory Coast and, secondarily, Cantanhez National Park in Guinea-Bissau. The opposing scenarios in terms of habitat fragmentation through deforestation, and the sharing of hunting as a common threat makes them ideal templates to assess primate communities' responses in different conditions.

Ivory Coast is part of the Upper Guinean Forests of West Africa biodiversity hotspot, a region that contains more than 2250 endemic plant species and 270 vertebrate species (Myers *et al*., 2000). This great number of vertebrates represents more than 25% of all African mammals, including more than twenty primate species (Myers *et al*., 2000), the second largest primate biodiversity in West Africa (Gonedelé Bi *et al*., 2012; Bitty *et al*., 2015). According to the IUCN (2016) the following species are native to Ivory Coast: *Piliocolobus badius*, *Piliocolobus waldronae*, *Colobus polykomos*, *Colobus vellerosus*, *Procolobus verus*, *Pan troglodytes verus*, *Cercopithecus diana*, *Cercocebus atys*, *Cercopithecus nictitans* Linnaeus, 1766; *Chlorocebus sabaeus* Linnaeus, 1766; *Galago senegalensis* É. Geoffroy Saint-Hilaire, 1796; *Erythrocebus patas* Schreber, 1774; *Cercocebus lunulatus* Temminck, 1853; *Cercopithecus campbelli* Waterhouse, 1838, *Cercopithecus lowei* Thomas, 1923; *Cercopithecus petaurista* Schreber, 1774; *Cercopithecus roloway* Schreber, 1774; *Galagoides demidoff* G. Fisher, 1806; *Galagoides thomasi* Elliot, 1907; *Papio anubis* Lesson, 1827; and *Perodicticus potto* P.L.S. Müller, 1766. However, the country has the biggest deforestation rate of all Sub-Saharan West Africa with an estimated 2650km^2 of forest annual loss (Gonedelé Bi *et al*., 2014). The small isolated forests that persist are currently threatened by illegal farming and logging (Gonedelé Bi *et al*., 2014). A study by Bitty *et al*. (2015) exposed that seven protected areas, inclusively Mont Péko and Marahoué National Parks, have been completely converted to agriculture, mostly for cocoa exploitation (Figure 1.9), a subject that has gathered substantial international awareness. It was also revealed a significant negative correlation between the area convert to cocoa fields and the presence of primates.

Figure 1.9 – Illegal activities conducted inside Ivory Coast's protected areas. On the left: cocoa plantation inside Niégré Forest Reserve; In the middle: illegal market inside Niégré Forest Reserve; On the right: primates hunted within Port-Gauthier Forest Reserve. From Bitty *et al*. 2015.

The Taï National Park (TNP) is, in general, an exception. Located in the Southwest of Ivory Coast (5°15'-6°7'N; 7°25'-7°54'W), the park is managed by the *Office Ivorien des Parcs et Réserves* (OIPR) and was classified as an UNESCO World Heritage Site in 1982 due to the extraordinary biodiversity and high number of endemic species it houses. These features justify its distinction as one of the 25 global biodiversity hotspots, included in the Upper Guinean Tropical Forests of West Africa, which once occupied a continuous and extensive area from Ghana to Sierra Leone (Myers *et al*., 2000). TNP covers an area of 5364 km^2 and is considered the biggest well preserved block of tropical forest in West Africa. According to the recent actualization of the Köppen-Geiger climate classification by Rubel and Kottek (2010) and long-term meteorological data (Servat *et al.*, 1997; Kühl *et al*., 2012), the TNP is located in an area classified as monsoonal and winter dry (savannah like) tropical/equatorial climate. The relative humidity in the park ranges between 85% and 90%, while the annual rainfall and temperature are 1800mm and 24ºC, respectively (Anderson *et al.* 2005). The designation of "block" gains a special relevance when we look at the its progressive isolation, as a result of the total deforestation of the surrounding areas (Figure 1.10; Brou Yao *et al*., 2005; Chatelain *et al*., 2010). Scientific research at the TNP started in 1979 with the creation of the Taï Chimpanzee Project and, 10 years later, the Taï Monkey Project. These projects are still ongoing and have proved to be very beneficial to the conservation of the primate populations in the park (Campbell *et al*., 2011), an aspect that expands its importance when we consider that 12 different species inhabit Taï's forests (Covey, 2009): *P. badius*, *C. polykomos*, *P. verus*, *P. t. verus*, *C. diana*, *C. atys*, *C. nictitans*, *C. petaurista*, *G. demidoff*, *G. thomasi* and *P. potto*. In 2005, the funding for research allowed the implementation of an effective monitoring programme (N'Goran *et al*., 2013) and, in 2014, it was created an ecotourism plan ("Nature and Culture") with the local community of Taï village that seeks to valorise the natural and cultural heritage, safeguarding the TNP while it generates simultaneously an economic contribution to the people. However, the TNP is not entirely threat-free: the anthropogenic activities, hunting above all, penalize daily the animal populations (Refisch and Koné, 2005a, 2005b; Covey, 2009; Hoppe-Dominik *et al*., 2011; N'Goran *et al*., 2013; Covey and McGraw, 2014), notwithstanding its prohibition by law in the whole country (Figure 1.10). Over 250000 kg of cercopithecid bushmeat are sold annually in the markets of the villages adjacent to TNP, leading to an overexploitation rate from 80% to 360% of several species (Refisch and Koné, 2005a, 2005b). Consequently, it has been noticed a sharp decline in the Ivorian primate populations in the last few decades. Briefly, the reasons supporting this decline are connected to the rapid growth of human population density, the influx of migrants, uncontrolled hunting and forest transformation for palm oil, rubber and cocoa production (Bitty *et al*., 2015). Among the affected species are *P. badius* and *C. polykomos* which, in 2012, following the study of Gonedelé Bi *et al*. were suggested to be categorized as endangered species in Ivory Coast.

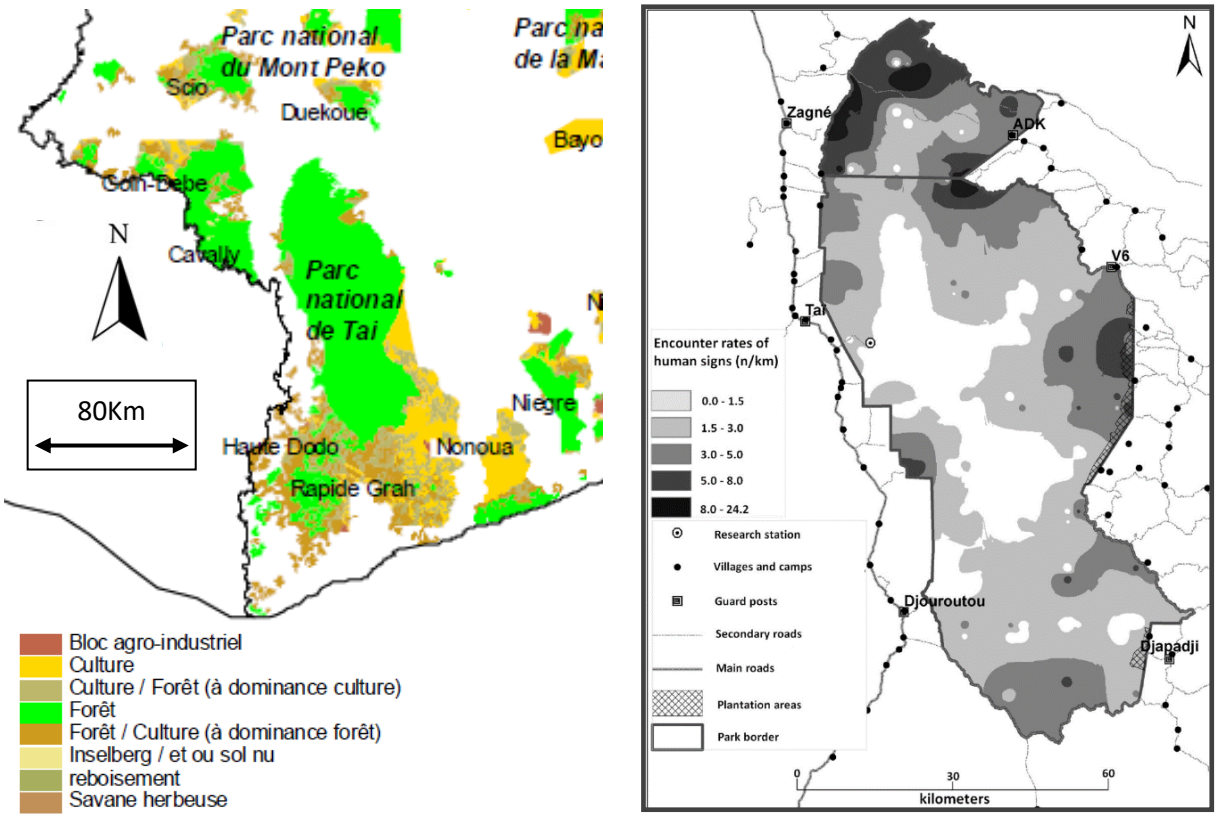


Figure 1.10 – On the left: Ivory Coast's protected areas – special emphasis on the TNP – and land use classification (adapted from Brou Yao *et al.*, 2005). Following the subtitle order: agro-industrial unit, agriculture, agriculture and forest (predominant agriculture), forest, forest and agriculture (predominant forest), bare ground, reforested ground, grassy savanna; On the right: spatial distribution of human presence signs in the TNP (from N'Goran *et al.*, 2013).

Guinea-Bissau is situated in West Africa and is classified as one of the world's 48 least development countries (UNCTAD, 2016). Agriculture represents the major activity in the country – especially cashew production (Temudo and Abrantes, 2014) – and its expansion walks side-by-side with deforestation. Between 2010 and 2015, Guinea-Bissau exhibited a deforestation rate of 10000 hectares per year (FAO, 2015). However, 16.7% of the country's terrestrial area is classified as protected area (UNEP-WCMC, 2016), managed by a national authority – the IBAP - *Instituto da Biodiversidade e das Áreas Protegidas* ("Institute for Biodiversity and Protected Areas") – and thus representing a governmental recognition of the potential benefits arising from a well-preserved natural patrimony. Even tough, the protected areas do not always represent an impenetrable shelter to its biodiversity. Hunting primates is prohibited in the whole country, but the law does not prevent its occurrence, especially in the southern region (Cá, 2008; Costa *et al.*, 2013; Hockings and Sousa, 2013). A study by Minhós and colleagues (2013a) estimated a consumption of 0.0032 primate carcasses per capita at the capital – Bissau – and confirmed the trade of 6 out of the 10 species of primates that inhabit the country. Gippoliti and Dell'Omo (1996, 2003) confirmed the presence of *P. temminckii*, *C. polykomos*, *C. campbelli*, *E. patas*, *C. sabaeus*, *Papio papio* Desmarest, 1820, *G. senegalensis*, *C. petaurista*, *C. atys* and *P. t. verus*. The first six species were encountered in Bissau's urban bushmeat markets.

The Cantanhez National Park (CNP), in the administrative region of Tombali, covers an area of 1058 km2 (11º 02‟ N; 15º 19‟ W) and was created in 2008 by a presidential decree, after reaching a consensus with governmental authorities and the local communities (around 23000 registered habitants

in 2014; IBAP, 2014) concerning in parallel the need to protect the biodiversity and the profit thereof could arise, especially through ecotourism (Gippoliti *et al.*, 2003; Hockings and Sousa, 2013). Cantanhez's forests represent the northern border of the Guinean sub-humid forests, with an exceptional biological diversity. CNP has over 200 bird species and nearly 100 mammal species, including 6 primate species (*P. temminckii*, *C. polykomos*, *C. campbelli*, *P. papio*, *C. sabaeus* and *P. t. verus*), of which 3 are considered to be threatened: the chimpanzees (endangered), the red colobus (endangered) and the black-and-white colobus (vulnerable) (Gippoliti and Dell'Omo, 2003; IBAP, 2014). Despite being managed by IBAP, the CNP management has been constrained by Guinea-Bissau's instability and economic status: even though it was legislated an interdiction on farming practices in certain forested sites, as well as hunting prohibition, the enforcement of the law was, unfortunately, limited by the lack of resources (Costa *et al.*, 2013; Sousa *et al.*, 2014). Recently, we found evidence of director's nomination for the CNP, as well as park rangers in indirect informal sources (such as a notice in a portuguese newspaper, or acknowledgments in scientific papers), but no official communication was available at the official IBAP website to this date. The director has already taken over and is currently working at the park (Maria Joana Ferreira da Silva personal communication in 2017). These news definitely represent a positive development for the CNP, and we have reasons to believe in the potential effectiveness of this novelty. CNP probably harbours what is the country's highest population densities of *P. temminckii* and *C. polykomos* known to date, however, this cannot be a synonymous of stability and prosperity of the species in the park. The genetic analysis of the populations and inference of the effective population size allowed to detect a recent bottleneck signal in both species (Figure 1.11), with a greater severity in *P. temminckii* that led to an effective population size inferior to 500 individuals (Minhós *et al.*, 2016). By dating this demographic event it was possible to conclude that it had an anthropogenic origin: habitat fragmentation, deforestation and hunting. These factors are remarkably visible in the CNP as reported in the land use map (Figure 1.12) and by Tânia Minhós personal communication in 2016.

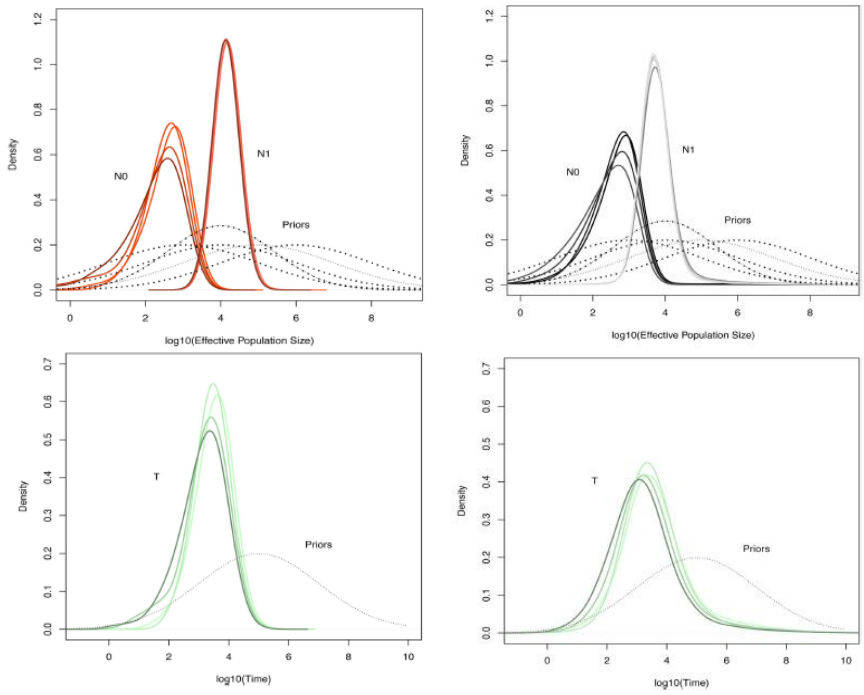


Figure 1.11 – Demographic bottleneck of *P. temminckii* (top left) and *C. polykomos* (top right), with the respective timing of its occurrence (below), in CNP using MSVAR 1.3. Top figures: solid lines - posterior distributions of current effective population size (N_0) and ancestral population size (N_1). Bottom figures: solid lines – posterior distribution for the time (in years) at which the bottleneck occurred. Dashed lines - prior distributions of the estimated parameters. All values are in a logarithmic scale. From Minhós *et al*. 2016.

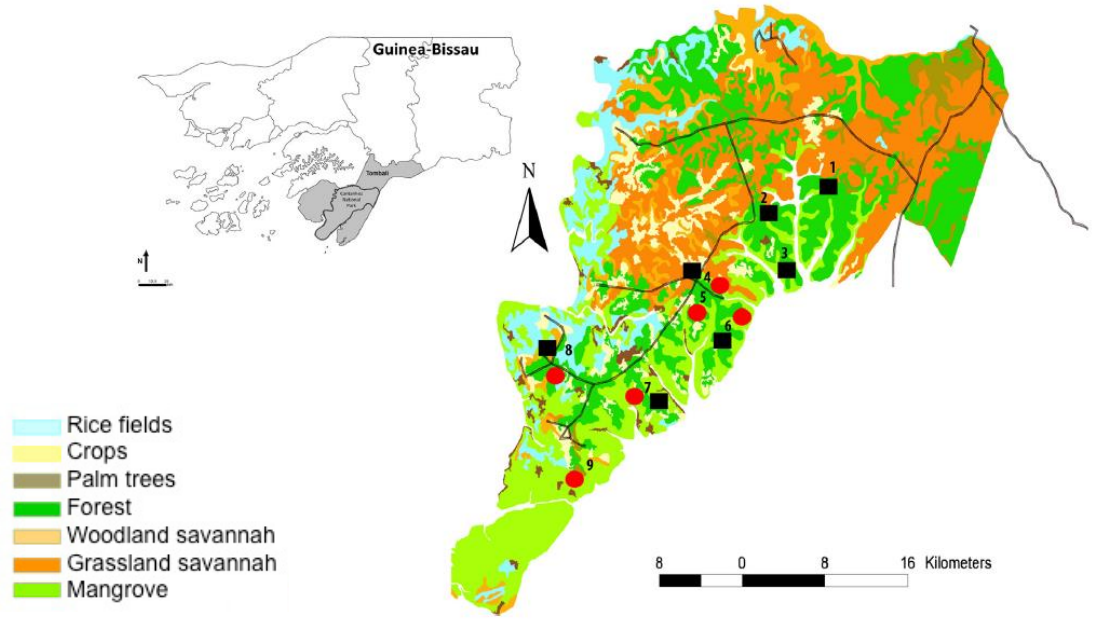


Figure 1.12 - Cantanhez National Park land use map. Adapted from Minhós *et al.* 2013b.

1.9 CONSERVATION AND FRAGMENTATION GENETICS

1.9.1 THE LINK BETWEEN GENETICS AND CONSERVATION

Genetic studies allow us to answer essential questions related to population and species' biology and ecology, when aiming for their conservation (Selkoe and Toonen, 2006). The conservation genetics (Frankham, 1995) field was born in this context and can be described as the area that uses genetic markers to quantify and study the world's genetic variability, contributing for the conservation of biodiversity (Shaffer *et al.*, 2015; Salgado-Lynn *et al.*, 2016). Among the most used genetic markers we can highlight the mitochondrial DNA (mtDNA) and the microsatellites. The latter – also named simple sequence repeats (SSR) or short tandem repeats (STR) – are very abundant in the nuclear DNA and, owing to this, became particularly popular in estimating contemporary migration, in distinguishing high migration rates from random mating systems, and to successfully solve kinship analysis between individuals (Selkoe and Toonen, 2006). Microsatellites are short sequences with 70 to 200 base pairs and a repetitive motif that varies between 1 and 6 base pairs, flanked by non-coding conservative DNA. In opposition to these regions, microsatellites are considered as ideal Mendelian markers – co-dominant markers – for being highly variable and by having the possibility of each locus present 5 or more alleles segregating in a single population.

Thanks to the growing number of described microsatellites and population genetic studies it has been possible to assess the status of several non-model threatened species. A review by Schwartz *et al.* back in 2006 highlighted the importance of the genetic monitoring of populations in conservation strategies, since DNA and population genetic data can provide valuable information, unattainable

through other approaches. Parameters like demography, the effective population size variation, population structure, kinship relations, inbreeding, hybridization, and the loss of genetic diversity are common and transversal to many studies (Allendorf *et al*., 2010). Although it is, sometimes, wrongly underestimated, the increasing number of threats imposes the species' genetic diversity analysis as a key factor to their conservation, especially when we consider an evolutionary timescale and the ability of species to adapt to environmental changes (Hoffman and Sgró, 2011; Salgado-Lynn *et al*., 2016). The loss of genetic diversity settles on a game between stochastic variables, condemn by the genetic drift. The genetic drift causes random changes in the alleles' frequencies over time and is responsible for the loss of heterozygosity and genetic variability, when an allele is fixed or lost. This effect is particularly severe in isolated populations with a reduced effective size, where the variation in the alleles' frequencies is more abrupt and the risk of fixation of a deleterious allele is higher. Therefore, demographic events like bottlenecks can have drastic consequences in populations and species' viability, and may even lead to its extinction (Frankham, 2005; Peery *et al*., 2012; Ellegren and Galtier, 2016). Its detection requires a careful exercise since there are other factors that induce signals of a decrease in the population size, like population structure (Chikhi *et al*., 2010). The use of genetic data allows us to infer the demographic history, helping in the detection of the factors (abiotic and biotic with a natural or anthropogenic origin) that once jeopardized the populations and may return in the future (Quéméré *et al*., 2012; Salmona *et al*., 2012; Malpica *et al*., 2014; Minhós *et al*., 2016).

In 2014, in a Radespiel and Bruford review article the concept of "fragmentation genetics" appeared for the first time. The term refers to the impacts caused by habitat fragmentation on populations, considering that it triggers an abrupt decrease in genetic diversity due to isolation and reduced gene flow, the increase of the inbreeding rate and the consequent decrease of the reproductive success rate (Marsh *et al*., 2013). Concerning primates, much has been done and achieved by analysing the genetic framework of populations (Vigilant and Guschansky, 2009): from population dynamics, to ecology, behaviour, social structure, conservation indications, among other attributes. In 2006, a pioneer study by Goossens *et al*. used genetic data to detect and quantify the impacts of anthropogenic habitat fragmentation and deforestation on the demographic collapse of the Bornean orangutan (*Pongo pygmaeus* Linnaeus, 1760). Dependent on the forest, primates are one of the first groups to suffer with these threats. Adding the significant menace posed by hunting, we have presented the reasons that are at the base of genetic studies that aim to contribute to primate species conservation.

1.9.2 POPULATION AND CONSERVATION GENETICS PARAMETERS

1.9.2.1 RELATEDNESS

The relatedness estimate between individuals is a common analysis in conservation genetics studies (Oliehoek *et al*., 2006). The relationship established between the individuals of a population may impact the standard genetic diversity estimates by transmitting a scenario in which the individuals genetically more similar than expected from a panmictic population (with direct consequences in the Hardy--Weinberg Equilibrium). Also, an increased relatedness due to population genetic structure, may raise a signal of spurious linkage associations between loci (Pritchard *et al*., 2000b). Our relatedness analysis aimed to control for any outcome that may be originated by this framework. In this sense, we performed a multi-software assessment and combined the results to create datasets reduced to the less related individuals (reduced datasets). Then, we re-run the previous analysis (described in sections 2.3.1, 2.3.2 and 2.3.3) to evaluate the possible effects.

1.9.2.2 SEX-BIASED DISPERSAL

Dispersal results in gene flow within and among populations. The manner and reasons by which individuals disperse across a landscape has a major impact on the genetic structure of a population, and thus the necessity of being considered in our study. Dispersal in primates is mostly mediated by males (Pusey and Packer, 1987), however, there are some exceptions, originated by different sex-derived behaviours that often represent a response towards ecological constraints, as intra-group competition for resources, mating and inbreeding avoidance (Handley and Perrin, 2007; Blyton *et al.*, 2015; Matsuda *et al.*, 2015). Without sex-specific markers to directly analyse male and female dispersal histories – such as Y-specific markers and mitochondrial DNA, respectively – or the possibility to compare pre--dispersing (often juveniles) and post-dispersing (often adults) individuals for each sex with autosomal markers (Vigilant and Guschanski, 2009), the sex-biased dispersal analysis falls upon indirect approaches as the one we used.

1.9.2.3 GENETIC DIVERSITY

The genetic diversity concept and its importance and connection to ecology, evolutionary and conservation biology was well covered in a review by Hughes *et al*. back in 2008. The genetic diversity of a population is equivalent to the amount of genetic variability that exists within the population's genetic pool. There is not a single way to estimate the genetic diversity of a population, but rather a group of measures that embrace not only the number of alleles that are present, but also their frequency and the frequency of the individual genotypes. Besides the quantitative and direct counting of the number of alleles per locus, the heterozygosity is a common metric of diversity and stands for the average proportion of loci that have two distinct alleles at a single locus per individual. Another as important and commonly used genetic diversity metric is the allelic richness. The allelic richness is measure that ponders not only the number of alleles per locus, but also their frequency, and thus is sensitive to the number of used samples. It is indicative of a population's capability to adapt and to persist – a population's evolutionary potential (Greenbaum *et al.*, 2016). Not all variation is related to an adaptive advantage, however, there is no standing genetic variation in the absence of allelic richness. Given this, it can be considered as an important tool in population conservation and management.

The expected frequencies of the alleles can be estimated by the basal principle of the Hardy--Weinberg Equilibrium, accepting an intrinsic group of assumptions (the organisms are diploid, exhibit sexual reproduction, do not have overlapping generations, mating occurs randomly and there is no genetic drift, nor migration, mutation, selection, or segregation distortion). There are four major forces controlling genetic diversity: selection, mutation, migration and genetic drift. Selection can either increase or remove genetic diversity, depending on the biotic and abiotic conditions that are influencing a population, it is the only force promoting evolutionary adaptation. Mutation is the source of all genetic diversity, and thus promotes its growth. Migration is responsible not only by increasing local variability through immigration, but also for homogenising populations. Finally, genetic drift is the sole force indorsing the removal of genetic diversity, as previously described. In our study, we accounted for the last two forces, migration over the dispersal analysis, and genetic drift by looking at *P. badius* and *C. polykomos* populations' genetic diversity in parallel with their demographic history.

1.9.2.4 POPULATION STRUCTURE

Population genetic structure refers to how genetic diversity is spatially distributed within a sampled population, or among subpopulations, where some individuals are genetically more identical to others, than to the remaining of the same species (Sethuraman, 2013). In primates, populations can become structured by several factors, such as social structure, the presence or absence of barriers to dispersal, different dispersal abilities, habitat segregation or demographic history (Vigilant and Guschanski, 2009; Sethuraman, 2013). Assuming that a population is not structured, or neglecting its existence, may compromise the remaining genetic-derived analysis and induce biased results and interpretations (Chikhi *et al*. 2010).

2 OBJECTIVES, HYPOTHESES AND PREDICTIONS

This study has three main objectives:

1. Characterize the genetic diversity and structure and infer the demographic history of *Piliocolobus badius* and *Colobus polykomos*' populations in Taï National Park, Ivory Coast;
2. Perform a comparative analysis between phylogenetically and ecologically similar – *Piliocolobus badius* and *Piliocolobus temminckii* – and conspecific – *Colobus polykomos* – populations from Cantanhez National Park, Guinea-Bissau;
3. Clarify the effects of habitat fragmentation and hunting on the genetic variability, evolutionary potential and, therefore, long-term viability of crucial and threatened primate populations.

Regarding the context, previous discoveries and the cited literature, we hypothesised and predicted the following:

1. TNP's forests still harbours genetically and ecologically healthy red colobus and black-and-white colobus populations.
    a. The alleged absence of physical barriers, and thus the presence of a large uninterrupted forest, will promote the maintenance of an elevated genetic diversity for both species, that does not compromise its evolutionary potential, without major comparable differences.
    b. The same habitat characteristics will promote the lack of structured populations.
    c. By ruling out contemporary fragmentation, the current hunting pressure will arise as an important explanation for a possible ongoing impoverishment of the genetic diversity.
    d. As these are recent threats, both species will present viable effective population sizes.
    e. The demographic history of both species can reflect the influence of past climatic oscillations and recent anthropogenic-derived variations of the effective population size.
2. TNP's populations should be more genetically diverse than CNP's populations.
    a. The higher genetic diversity levels of TNP's populations will be promoted by both contemporary and ancestral stability of the forests, and weaker anthropogenic pressures.
    b. In the absence of habitat fragmentation, the red colobus and black-and-white colobus populations will more easily accommodate the hunting pressure.
    c. The combined effects of habitat fragmentation and hunting will pose a greater threat towards the colobine populations.

As final notes, we hope that our study provides scientific evidence to support or raise any necessary conservation measures for TNP's red colobus and black-and-white colobus populations, but also for CNP's populations. We think our study will strengthen the warning signal towards CNP's colobines and the need to safeguard all the remaining individuals to ensure a future for the local populations. The genetically and ecologically healthy populations we hope to find at TNP's forests will help to quantify and characterize any deviations in other red colobus and black-and-white colobus populations. As forest-dependent primates, our results may be taken in account in other studies with similar models.

3 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION AND DNA EXTRACTION

We analysed 29 DNA samples of *Piliocolobus badius* and 8 DNA samples of *Colobus polykomos* (Table AT 1), obtained by Tânia Minhós from IGC through a collaboration with Sebastien Calvignac--Spencer of the Robert Koch Institute (Berlin, Germany), whose team gathered all the samples and conducted the DNA extraction. These tissue samples were collected from carcasses or darted specimens found in several surveys, carried between 2004 and 2010 at the TNP (approximate central point of the surveyed area: 5º50.34'N -7º19.26'W; Calvignac-Spencer *et al*., 2013; Schubert *et al*., 2015). According to the cited authors, DNA was extracted from tissue using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total DNA concentrations of all extracts were measured with a Nanodrop device (Thermo Scientific, Waltham, MA, USA).

3.2 LAB PROCEDURES

3.2.1 CARRION FLY-DERIVED PRIMATE NUCLEAR DNA AMPLIFICATION

The collaboration with Sebastien Calvignac-Spencer of the Robert Koch Institute (Berlin, Germany) has also allowed us to conduct another experiment regarding the primate DNA extraction from carrion flies. Using four previously extracted nucleic acid samples from carrion flies – which we already knew had *P. badius* and *Colobus polykomos* DNA due to previous analysis – we accessed the possibility of genotyping these individuals with some of the reported microsatellites. Again, the sample collection and DNA extraction were performed by the Calvignac-Spencer's team of the Robert Koch Institute (Berlin, Germany). The samples, in this case the carrion flies, were gathered at the TNP by using a mosquito net trap and a bowl containing either a piece of meat or a commercial fly bait (Calvignac-Spencer *et al*., 2013; Schubert *et al*., 2015). Afterwards the flies were placed in tubes and snap-frozen in liquid nitrogen immediately after being collected. Regarding the DNA extraction, carrion flies were cut into small pieces using sterilized scissors and submerged in FastPrep® lysing matrix tubes (MP Biomedicals, Illkirch, France) with 250µL phosphate buffer saline. Then the tubes were agitated in a FastPrep® system. Finally, nucleic acids were extracted from 100µL fly mixture using the GeneMATRIX Stool DNA Purification Kit (Roboklon, Berlin, Germany) following the manufacturer's instructions (Calvignac-Spencer *et al*. 2013; Schubert *et al*. 2015).

The samples we used were analysed trough mitochondrial DNA (mtDNA) specific sequencing and next-generation sequencing in order to identify the species of mammals present in the carrion carnivorous flies (Calvignac-Spencer *et al*., 2013; Schubert *et al*., 2015). In our case, they contained DNA from *P. badius* and *C. polykomos*. Our challenge was to amplify nuclear DNA, which occurs in much less quantity than mtDNA in our cells. From the list of microsatellites used before, we chose the ones that provided the better and clearer outcomes in terms of electropherogram interpretation. D4s2408 was pointed for a PCR singleplex with 2.5µL Multiplex PCR Master Mix (Qiagen), 0.25µL of the

primer, 0.1µL of Q solution (Qiagen; Q solution has the same function as the BSA in stabilizing the reaction), 2µL of template DNA and 0,15µL of ultrapure water, in a final volume of 5µL. For positive control we used a sample that contained DNA from *Colobus polykomos*. The protocol for the reactions, including negative and positive controls, was: 15 minutes at 95ºC; 40 cycles of 0.5 minutes at 94ºC, 1.5 minutes at 53-59ºC, 1.5 minutes at 72 ºC followed by 30 minutes at 72ºC; 12 minutes at 4ºC. Given the negative results of this experiment, we tried two new PCR singleplex with two other primers, D13s321 and D4s2408, following exactly the same protocols, with two exceptions: the number of amplification cycles that were increased from 40 to 60 in both reactions; in D4s2408 singleplex we fixed the optimum annealing temperature in 57ºC, the reported optimum.

3.2.2 DNA AMPLIFICATION, MICROSATELLITE GENOTYPING AND ALLELE SCORING

All samples were genotyped for 15 human-derived microsatellite loci – successfully used for the first time by Harris *et al.* (2009) in *Colobus guereza* – and multiplexed in three panels of five loci. Molecular sex identification of each sample was carried out following the protocol by Villesen and Fredsted (2006) and multiplexed together with the microsatellites (Table 3.1). Tested in a wide range of primate taxa – with overall positive results – the protocol's key for sexing primate DNA relies on the X chromosome linked DEAD-box polypeptide three gene (DDX3X). In the studied colobine species, this primer has a fragment size of 178 bp on the X chromosome and 210 bp on Y. Except for the DEAD--box gene, in which being homozygous indicates that the individual is a female and the heterozygous a male, all the remaining microsatellites are characterized by a tetranucleotidic motif. Primers sequences can be consulted in Table AT 2.

Table 3.1 - Primers used in the study.

Multiplex	Primer/Locus	Annealing Temperature in Singleplex PCR (ºC)
1	**D2s1326**	56
	D4s2408	57
	D6s474	60
	D10s611	55
	D13s321	57
2	**D1s548**	55
	D2s442	55
	D11s2002	64
	D12s372	55
	Fesps	57
3	**D1s1665**	57
	D6s503	55
	D6s1056	55
	D10s676	60
	D10s1432	56
	DEAD-box	57

The polymerase chain reactions (PCRs) with the reported primer panels followed the protocol optimized in Minhós *et al*. (2013b) with few optimization steps, essentially required due to the use of a different kind of samples: Minhós *et al*. (2013b) has used low quality DNA from faecal samples and, on this project, we have used high quality DNA from tissue samples. The most relevant adjustments were the removal of the BSA (Bovine Serum Albumin, NEB) – a protein that helps in the stability of the reaction –, and the reduction of the number of amplification cycles from 40 to 25. We tested the performance of two kits, Qiagen and MyTaq, with 14 samples (including two positive plus two negative controls) – through an agarose gel electrophoresis and allele scoring methodologies – to evaluate which one produced better results. At the end, we chose Qiagen. PCRs contained 5μL Multiplex PCR Master Mix (Qiagen), 0.1μL of each primer (a total of 0.5μL of the primer mix), 1.0μL of template DNA and 3.5μL of ultrapure water, in a final volume of 10μL. The reactions, including negative and positive controls, were performed in a BIORAD efx95r PCR machine (Applied Biosystems) as follows: 15 minutes at 95ºC; 25 cycles of 0.5 minutes at 94ºC, 1.5 minutes at 55-57ºC, 1.5 minutes at 72 ºC followed by 30 minutes at 72ºC. A final step of 12 minutes at 4ºC was added to assure the end of the PCR reactions. PCR products were analysed by the IGC's Genomics Unit in an ABI 3130XL Automatic Sequencer. An internal size standard ladder was added, after testing the performance of Rox500 and LIZ, the latter was chosen since it produced better results.

Alleles were scored using GENEMAPPER® Software version 4.1 (Applied Biosystems). The genotypes were considered as heterozygous after we observed each allele in at least two independent PCR reactions, the minimum acceptable number of repetitions used as criteria to validate the genotypes of all samples owing the quality of the DNA. The alleles were scored independently twice. The loci D10s611, D1s548, D12s372 and D10s1432 were excluded from the dataset of *P. badius* as genotypes were missing or declared invalid (per example by not respecting the microsatellite motif or by not fitting the established genotyping criteria) for the majority or even all the samples. For the same reasons the loci D10s611, D12s372 and D10s1432 were also excluded from *C. polykomos*' dataset. Given our results, the locus D10s611, which had previously provided a good outcome in the amplifications by Minhós *et al*. (2013b) and subsequent studies, was isolated in a singleplex with the samples that had yielded the clearer electropherograms for several loci before. PCRs contained 0.1μL Taq polymerase, 1.2μL $MgCl_2$, 0.9μL dNTPs, 0.8μL of the primer mix (forward and reverse), 2μL of buffer solution, 4μL of ultrapure water and 1μL of template DNA. The only difference to the already cited reaction' conditions was the increase of the amplification cycles from 25 to 40. However, even though we have increased the quantity of $MgCl_2$ in the reaction (to increase the stability) and the number of amplification cycles, following Arandjolovic *et al*. (2009) recommendations, there was no amplification at all.

A total number of 830 PCR reactions were performed in this study.

3.3 SOFTWARE ANALYSIS

3.3.1 QUALITY CONTROL OF GENETIC DATA

3.3.1.1 STANDARD GENOTYPING PROCEDURES

The probability of identity (PI_{sibs}, the probability of observing identical multilocus genotypes between two individuals sampled froma population where close related individuals are included) values

by locus – that accounts for the presence of related individuals in the sample (Waits *et al.*, 2001) –, were estimated in GENALEX 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012). At this stage, the minimum number of successfully and accepted genotyped loci per sample was above 63% (7/11 microsatellite loci) and 91% (11/12 microsatellite loci) for *P. badius* and *C. polykomos*, respectively. To identify genotyping errors due to null alleles, short allele dominance (large allele dropout) and scoring errors due to stuttering we have used the software MICROCHECKER (van Oosterhout *et al.*, 2004). The presence of null alleles was later assessed also in ML-RELATE (Kalinowski *et al*., 2006). The departures from the Hardy-Weinberg Equilibrium (HWE) and the extent linkage disequilibrium (LD) analysis between pairs of loci were calculated in GENALEX 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012) and GENEPOP (Raymond and Rousset, 1995; Rousset, 2008), with dememorization of 10000, 1000 batches and 10000 iterations for the Markov chain Monte Carlo method (MCMC). This double-software analysis was conducted to evaluate the possibility of different outcomes and interpretations of the values. We also applied the Bonferroni correction for multiple simultaneous comparisons and, due to its severity (Narum, 2006), the more robust false discovery rate correction method based on the algorithm proposed by Benjamini and Hochberg (1995) (FDRBH). The concept of the FDRBH is to use, as measure of global error, the expected proportion of false rejections of H_0 (type I errors) between the overall rejections, the "true" results. This allows an estimation of a threshold value that fits our dataset: if higher proportion of the tested H_0 is truly false, the FDRBH will find a lower threshold value than the Bonferroni correction (Sabatti *et al.*, 2003). Similar to the p-values significance levels, we have also used 0.05, 0.01 and 0.001 as *a priori* values for the FDRBH. The spreadsheets used for these corrections were created and made available online by McDonald (2014). The HWE and LD analysis were conducted for two datasets, first with all the individuals (full datasets) and second with the less related individuals (reduced datasets). The coefficient of inbreeding (F_{IS}) was estimated following the method by Weir and Cockerham (1984) in FSTAT (Goudet, 2001). Two pairs of individuals of *P. badius* were identified as being the same after running the multilocus matches analysis in GENALEX 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012). Considering the sampling scheme and the fact that these samples were collected in different years, they could not correspond to the same individual. Consequently, we have decided to include them all in the *P. badius*' full dataset. Excluding one of these individuals could bias the genetic diversity data, especially the allelic richness, which has in account not only the number of different alleles, but also their frequency in the population. This aspect has been addressed in the next section.

3.3.1.2 RELATEDNESS

The mean pairwise relatedness analysis – the estimation of genotypic identity-by-descent (IBD) – was firstly assessed using the package RELATED (Pew *et al*., 2015) implemented in R version 3.0.2 (R Core Team, 2013). The underlying code of this package is the same as the software COANCESTRY version 1.0.1.5 (Wang, 2011). The analysis (200 replicates) of our dataset with the Pearson correlation coefficients between the observed and expected values for several relatedness estimators – Lynch and Li (LL; Lynch, 1988; Li *et al*., 1993), Lynch and Ritland (LR; Lynch and Ritland, 1999), Queller and Goodnight (QG; Queller and Goodnight, 1989) and Wang (W, Wang, 2002) – allowed us to have an idea of which one fitted best our data (Table 4.4). After that, we used COANCESTRY version 1.0.1.5 (Wang, 2011) to obtain the summary statistics of the relatedness point estimates and the correlations coefficients between the different estimators (Table 4.5). The performance of marker-based estimators has been demonstrated to be affected by the relatedness composition of the studied population (Csilléry *et al*., 2006), the number and polymorphism of the used loci (Blouin, 2003), as well as the demographic history of the population (Taylor, 2015). We chose Wang's estimator (Wang, 2002), based in our results.

Since we identified the presence of null alleles in some of our loci, we also used ML-RELATE (Kalinowski *et al*., 2006) for a more direct approach on this issue. The detection of null alleles through an Hardy-Weinberg test for heterozygote deficiency was conducted with 10000 MCMC randomizations (Guo and Thompson, 1992) and the 'U' test statistic described by Rousset and Raymond (1995). We estimated the relationship between the individuals with 95% confidence intervals for a 0.05 level of significance, with 10000 randomizations, under custom relatedness values (r) criteria: r=0.5 for parent-offspring (PO) and full sibs (FS), r=0,25 for half-sibs (HS) and r=0 for unrelated (UN) individuals. Finally, we used KINGROUP v2_0909501 (Konovalov *et al*. 2004) to test for the significance of the mean pairwise relatedness under Wang's estimator (Wang, 2002). Between pairs with an r value equal or superior to 0.25 and a p-value inferior to 0.05, one of the members was discarded based on its genotyping success and the established number of relationships with other individuals.

We verified that, in order to remove all the related individuals in the datasets, our sample size would decrease significantly. This statement justifies the application of our multi-source criteria, which allowed the maintenance of some individuals, without compromising the robustness of the analysis. The reduced dataset of *P. badius* contained 22 individuals, while *C. polykomos*' contained 6 individuals. After comparing the full with the reduced datasets, we decided to proceed with the full datasets for both species since the results were equal or even negatively affected by the individuals' removal. In this study, all analysis were further conducted with the full datasets for both species.

3.3.2 SEX-BIASED DISPERSAL

The mean corrected assignment indices (mAI_c) were estimated and compared between the two sexes using GENALEX 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012), that follows the method of Mossman and Wasser (1999). The assignment test refers to the probability of an individual's genotypic composition fit the overall sampled population. With log-likelihoods as negative values, the least negative (highest) value corresponds to the highest probability of belonging to the population. In this sense, due to the mAI_c correction for zero, the immigrant genotypes should present negative values (less likely to belong to the population), while positive values should designate natal individuals. Two important aspects to have in account are: (1) microsatellite-derived genetic data allows us to obtain evidence of the direction of instantaneous sex-biased dispersal (Handley and Perrin, 2007); (2) a sex-biased dispersal – in case of existing –, evaluated solely through a mAI_c analysis, must be intense enough to be detected by such populational methods (Goudet *et al.*, 2002). We were unable to distinguish between post-dispersal (adults) and pre-dispersal individuals (juveniles) in our data. This stage differentiation is a common assumption in indirect sex-biased dispersal analysis, since it empowers the detection of uncommon alleles brought to the population through immigration: if only one sex disperses, its genes are transmitted in the next generation to both sexes (Goudet *et al*., 2002). Our indirect approach to this issue was steered due to the effect of dispersal on gene flow and, consequently, population's genetic structure (Montague *et al*., 2014; Milton *et al*., 2016).

3.3.3 GENETIC DIVERSITY

The number of alleles (Na) and effective alleles (Ne; 1/(Σ pi^2, where pi is the frequency of the ith allele for the population and Σ pi^2 is the sum of the squared population allele frequencies), and the

observed (Ho; Number of Heterozygotes/N) and unbiased expected heterozygosity (uHe; He*(2N/(2N-1))) were estimated using GENALEX 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012).

The uneven allelic richness for the populations at the TNP was estimated in FSTAT (Goudet, 2001) and HP-RARE (Kalinowski, 2005) and then compared with the values obtained by Minhós *et al*. (2013b). The mean total number of alleles across all loci was estimated by fitting cumulatively the rarefaction value for each individual. The rarefaction method uses the frequency distribution of alleles at a locus to obtain the number of alleles that would be present in a smaller set of samples (Leberg, 2002). Therefore, is the most suitable and commonly used approach when aiming to compare populations with unequal sample sizes (Basto *et al*., 2016; Zanin *et al*., 2016). The logarithmic tendency lines were all extrapolated to 71 samples to give a visual idea of allelic richness' cumulative growth rate until the compared maximum number of samples.

3.3.4 POPULATION STRUCTURE

The population genetic structure was assessed using a Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard *et al*., 2000a) without a fixed *a priori* spatial assignment, in order to detect the given number of genetic clusters (K). K varied between 1 and 5, using five independent runs, with as initial 100000 burn-in period and 1000000 MCMC iterations. The parameters set were based on an admixture model with correlated allele frequencies. We used STRUCTURE HARVESTER (Earl and vonHoldt, 2012) online to infer the most fitting K by Evanno's ΔK method (Evanno *et al*., 2005), which finds the uppermost level of structure in a given dataset through an *ad hoc* summary statistic based on the slope of variation of the estimated likelihood between sequential K values. Evanno's ΔK method is incapable of validating a scenario where there is only 1 genetic cluster (K=1; Evanno *et al*., 2005), since the slope is inexistent. Conscious of this, we followed a commonly approach to face this limitation (Coulon *et al*., 2008; Gilbert *et al*., 2012; Quintela *et al*., 2014): the analysis of the Ln P(D) outputs provided by STRUCTURE 2.3.4 (Pritchard *et al*., 2000a), that are converted to L(K) by Evanno's method (Evanno *et al*., 2005). The graphical outcomes of this approach were also provided by STRUCTURE HARVESTER (Earl and vonHoldt, 2012). We have also conducted a two-dimensional factorial correspondence analysis (FCA) in GENETIX 4.05 (Belkhir *et al*., 2004) for both species, showing the distribution of the individual genotypes according to the axes.

3.3.5 DEMOGRAPHIC HISTORY

The demographic history of both species was analysed through three different methods. First, we aimed to detect any signal of heterozygosity excess. The heterozygosity excess at selectively neutral loci – that occurs when the observed heterozygosity is higher than expected from a population at mutation-drift equilibrium – is indicative of a decrease in the effective population size (*i.e.* a bottleneck, or that heterozygotes exhibit a selective advantage) (Cornuet and Luikart, 1996). This excess is originated by the faster decline of the number of alleles than heterozygosity: a genetic bottleneck leads to the removal of low-frequency/rare alleles and this effect occurs earlier and more abruptly than any variation in heterozygosity (Nei *et al*., 1975). In order to conduct this analysis, we ran the software BOTTLENECK (Cornuet and Luikart, 1996; Piry *et al*., 1999) and applied the Wilcoxon's sign-rank (Luikart *et al*., 1998) test with 10000 replications for the three mutation models: infinite allele model

(IAM), stepwise mutation model (SMM) and the two phase-model (TPM). TPM ran with 30% variance and 70% proportion of SMM.

The posterior distributions of the demographic parameters were estimated through MCMC simulations in MSVAR 1.3 (Storz and Beaumont, 2002). This hierarchical Bayesian method assumes a simple demographic model with only one population effective size change and estimates the current and ancestral population sizes (N_0 and N_1, respectively) and the time (T) in generations since the population size started to vary, assuming an exponential size change. The model adopts lognormal prior distributions for the parameters N_0, N_1, T and μ and that microsatellites are evolving under SMM. The generation time for both species was set as 5 years (Allen *et al*., 2012; Minhós *et al*., 2016). The simulations ran under 5 distinct independent scenarios: population stability (scenario 1: $N_0=N_1$), severe demographic bottleneck (scenario 2: $N_0<<N_1$), great demographic expansion (scenario 3:$N_0>>N_1$), small demographic expansion (scenario 4: $N_0>N_1$) and small demographic bottleneck (scenario 5: $N_0<N_1$). This was made by varying the priors and hyperpriors distributions of each run (Table AT 3). All runs were conducted with 300000 thinned update steps and a 30000 thinning interval. We removed the first 10% of each independent simulation (burn-in) to avoid any implications of the starting conditions in our parameter estimations. The Brooks, Gelman and Rubin Convergence Diagnostic test (Gelman and Rubin, 1992; Brooks and Gelman, 1998) was used to assess the convergence between the scenarios, in addition to the visual evaluation. The stated diagnostic test evaluates the MCMC convergence by analysing the difference between multiple Markov chains, in the form of a multivariate potential scale reduction factor (MPSRF; Brooks and Gelman, 1998). The convergence is assessed by comparing the estimated between- and within-chains variances for a set of variables. Only the second half of chains is used in the estimation. As chains converge to a common final distribution, the between-chain variability should become small relative to the within-chain variability, resulting in a MPSRF close to 1. A 0.975 quantile greater than 1.20 is considered as evidence of absence of convergence. The test was conducted in the package BOA version 1.1.7-2 (Smith, 2007) implemented in R version 3.0.2 (R Core Team, 2013). The highest probability density intervals were also estimated for $\alpha = 0.05$ using the same package.

Recent variations in the effective population size were evaluated using the package VAREFF (Nikolic and Chevalet, 2014) implemented in R version 3.0.2 (R Core Team, 2013). It is worth saying that VAREFF represents a novel approach that, until this moment, has only been utilized four times in published scientific papers (Perrier *et al*., 2013; Henriques *et al*., 2016; Lourenço *et al*., 2017; Rochus and Johansson, 2017). VAREFF relies on the hypothesis that all markers follow the same mutation model and have the same mutation rate. An advantage over MSVAR 1.3 (Storz and Beaumont, 2002) is the considerable independence on the established priors and the possibility to explore the dependence on the chosen mutation model (Nikolic and Chevalet, 2014). We ran a preliminary analysis to obtain the most appropriate values for the Theta priors, as well as an exploratory assessment of the consequences of changes in the parameters. The preliminary runs allowed us to obtain the best estimates of Theta correlated to the mean of present (Theta0), intermediate (Theta1) and ancestral (Theta2) population sizes. It also provided the imbalance indices (I1=ln[Theta1/Theta0]; I2=ln[Theta2/Theta0]) and the minimum (N_0=Theta0/4*mutation rate) and maximum (N_1=Theta2/4*mutation rate) effective population sizes. The parameters were the following: number of loci (NBLOC = 11 for *C. polykomos* and NBLOC = 10 for *P. badius*); number of times Ne has changed trough time (JMAX = 2); mutation model (MODEL = SMM); mutation rate (MUTAT = 0.001); global prior mean of effective size (NBAR = 10000); variance of the prior log-distribution of effective size (VARP1 = 3, as suggested); coefficient of correlation between effective sizes in successive intervals (RHOCORN = 0, as suggested); number of generations since the assumed origin of population (GBAR = 100000); variance of the prior log--distribution of time intervals where the population size is assumed to be constant (VARP2 = 3);

maximal distance between alleles (DMAX = 8 for *C. polykomos* and DMAX = 14 for *P. badius*); and a smoothing parameter (Diagonale = 0.5, as suggested). The simulations ran with 10000 MCMC steps, with a length of 1000, space of 10, burn-in period of 10000 (1 million iterations) and an acceptation rate of 0.25 (as suggested).

The results from MSVAR 1.3 (Storz and Beaumont, 2002) and VAREFF (Nikolic and Chevalet, 2014) were validated by running two subsets of individuals from CNP with a sample size equal to TNP's species (8 for *C. polykomos* and 29 for *P. badius*).

4 RESULTS

4.1 CARRION FLY-DERIVED PRIMATE NUCLEAR DNA AMPLIFICATION

Given the negative results in all trials of amplifying carrion fly-derived primate nuclear DNA – due to the low quantity and quality of the DNA - and the resources already spent, this part of the project was, unfortunately, abandoned. Being successful would have allowed us, on one hand, to create a new protocol that nobody has ever used before and generate a huge amount of scanning and sampling possibilities based on carrion-fly DNA (environmental DNA, eDNA) under several contexts and, on the other hand, to increase our sample number in this project.

4.2 QUALITY CONTROL OF GENETIC DATA

4.2.1 STANDARD GENOTYPING PROCEDURES

The PI_{sibs} analysis reported the acceptable values of 1.5×10^{-5} for *P. badius* and 2.1×10^{-5} for *C. polykomos*. All loci used were polymorphic. *P. badius* dataset contained 11 males and 16 females, while *C. polykomos* dataset had 3 males and 3 females. ML-RELATE reported the presence of null alleles in locus D6s474 for *P. badius* and in loci D1s548, D13s321, D2s1326, D11s2002, D6s1056, D10s676 for *C. polykomos*. MICROCHECKER corroborated the results for *P. badius*, and detected the presence of null alleles in loci D11s2002 and D10s676 for *C. polykomos* (Table AT 4). We opted to consider the presence of null alleles in loci that had such results in both software: D6s474 in *P. badius*, and D11s2002, plus D10s676 in *C. polykomos*. From the two software used to assess the departures from the Hardy-Weinberg equilibrium (HWE) and the extent linkage disequilibrium (LD) analysis between pairs of loci we chose to rely on GENEPOP's results (Tables 4.1 and 4.2). The consistent results obtained after applying both the Bonferroni correction and the false discovery rate correction method based on the algorithm proposed by Benjamini and Hochberg (1995) supported this choice. After the corrections, the loci D2s442 (p-value<0.001) and D10s676 (p-value<0.001) – in the respective species – continued to show a significant departure from HWE. In terms of LD, we found a significant relationship between loci D10s676 and D6s503 in *P. badius* (p-value=0.000; Table 4.3). Therefore, the loci D2s442 and D10s676 were removed from the datasets of *P. badius* and *C. polykomos*, respectively, and unused in further analyses. We decided to keep the loci D10s676 and D6s503 in *P. badius* dataset since no other issues were encountered. Relatively to locus D6s474 in P. badius, we decided to maintain it in our dataset, since we did not expect any major effect in our focal analysis (Dakin and Avise, 2004; Chapuis and Estoup, 2007; Carlsson, 2008; Chybicki and Burczyk, 2009).

Table 4.1 - Hardy-Weinberg Equilibrium analysis in *P.badius*. ns = not significant; * P<0.05; ** P<0.01; *** P<0.001. SE = standard error; BC = significance after the Bonferroni correction; BHC = significance after applying the false discovery rate method of Benjamini and Hochberg (1995). Results from GENALEX and GENEPOP.

	Piliocolobus badius										
	GENALEX						GENEPOP				
Locus	**DF**	**ChiSq**	**p-value**	**Signif**	**BC**	**BHC**	**p-value**	**SE**	**Signif**	**BC**	**BHC**
D1s1665	55	80.141	0.015	*	ns	*	0.3184	0.0043	ns	ns	ns
D4s2408	36	24.056	0.936	ns	ns	ns	0.9790	0.0003	ns	ns	ns
D13s321	45	36.293	0.819	ns	ns	ns	0.2731	0.0027	ns	ns	ns
D6s474	45	82.594	0.001	***	*	**	0.6608	0.0027	ns	ns	ns
D2s1326	78	118.974	0.002	**	*	**	0.1294	0.0034	ns	ns	ns
Fesps	28	38.682	0.086	ns	ns	ns	0.3879	0.0020	ns	ns	ns
D11s2002	45	29.214	0.967	ns	ns	ns	0.6985	0.0020	ns	ns	ns
D2s442	78	155.417	0.000	***	***	***	0.0000	0.0000	***	***	***
D6s503	66	77.589	0.156	ns	ns	ns	0.1131	0.0021	ns	ns	ns
D6s1056	21	24.357	0.276	ns	ns	ns	0.2959	0.0012	ns	ns	ns
D10s676	36	38.632	0.352	ns	ns	ns	0.2374	0.0017	ns	ns	ns
							All (Fisher's method): Chi2: 48.8193; Df: 22.0000; Prob: 0.0008				

Table 4.2 - Hardy-Weinberg Equilibrium analysis in *C. polykomos*. ns = not significant; * P<0.05; ** P<0.01; *** P<0.001. SE = standard error; BC = significance after the Bonferroni correction; BHC = significance after applying the false discovery rate method of Benjamini and Hochberg (1995). Results from GENALEX and GENEPOP.

	Colobus polykomos										
	GENALEX						GENEPOP				
Locus	**DF**	**ChiSq**	**p-value**	**Signif**	**BC**	**BHC**	**p-value**	**SE**	**Signif**	**BC**	**BHC**
D1s548	15	26.667	0.032	*	ns	ns	0.0144	0.0004	*	ns	ns
D1s1665	10	8.327	0.597	ns	ns	ns	0.4908	0.001	ns	ns	ns
D4s2408	6	2.436	0.876	ns	ns	ns	1.0000	0	ns	ns	ns
D13s321	6	2.852	0.827	ns	ns	ns	0.6650	0.0009	ns	ns	ns
D6s474	21	29.111	0.111	ns	ns	ns	0.5288	0.0016	ns	ns	ns
D2s1326	10	17.333	0.067	ns	ns	ns	0.2591	0.0009	ns	ns	ns
Fesps	6	3.440	0.752	ns	ns	ns	1.0000	0	ns	ns	ns
D11s2002	28	37.000	0.119	ns	ns	ns	0.0315	0.0008	*	ns	ns
D2s442	21	28.444	0.128	ns	ns	ns	0.6210	0.0015	ns	ns	ns
D6s503	28	27.111	0.512	ns	ns	ns	0.6934	0.0018	ns	ns	ns
D6s1056	6	9.481	0.148	ns	ns	ns	0.2627	0.007	ns	ns	ns
D10s676	6	16.163	0.013	*	ns	ns	0.0005	0	***	**	**
							All (Fisher's method): Chi2: 42.5044; Df: 26.0000; Prob: 0.0218				

Table 4.3 - Genotypic linkage disequilibrium analysis (log likelihood ratio statistic) in *P. badius* (p-values shown in the upper part of the table) and *C. polykomos* (p-values shown in the lower part of the table). NI=No information. * Significance for p-values under 0.05 after the Bonferroni correction. Results provided by Genepop.

	Piliocolobus badius											
Locus	**D1s548**	**D1s1665**	**D4s2408**	**D13s321**	**D6s474**	**D2s1326**	**Fesps**	**D11s2002**	**D2s442**	**D6s503**	**D6s1056**	**D10s676**
D1s548												
D1s1665	1.000		0.414	0.360	0.135	0.010	0.352	0.122	0.170	0.112	0.228	0.191
D4s2408	0.107	1.000		0.026	0.062	0.123	0.173	0.025	0.002	0.047	0.113	0.123
D13s321	0.430	1.000	0.142		0.320	0.511	0.278	0.139	0.151	0.044	0.490	0.039
D6s474	1.000	1.000	1.000	0.285		0.068	0.363	0.083	0.103	0.147	0.002	0.311
D2s1326	1.000	0.200	1.000	1.000	1.000		0.002	0.122	0.146	0.045	0.148	0.466
Fesps	0.200	1.000	1.000	1.000	1.000	1.000		0.163	0.191	0.077	0.221	0.063
D11s2002	NI	NI	NI	NI	NI	NI	NI		0.039	0.036	0.029	0.130
D2s442	0.200	1.000	1.000	1.000	1.000	1.000	1.000	NI		0.054	0.165	0.152
D6s503	NI	NI	NI	NI	NI	NI	NI	NI	NI		0.236	0.000*
D6s1056	0.386	1.000	0.145	0.514	0.275	1.000	1.000	NI	0.271	NI		0.141
D10s676	0.443	1.000	1.000	1.000	1.000	1.000	1.000	NI	0.324	NI	1.000	
Locus	**D1s548**	**D1s1665**	**D4s2408**	**D13s321**	**D6s474**	**D2s1326**	**Fesps**	**D11s2002**	**D2s442**	**D6s503**	**D6s1056**	**D10s676**
	Colobus polykomos											

4.2.2 RELATEDNESS

Overall, the mean pairwise relatedness estimator that fitted best our data was Wang's (2002) (Table 4.4). However, the regression-based method of the moments estimator of LR provided the best correlation for the *C. polykomos* dataset (0.822). Nonetheless, this result could be biased by LR's weaknesses: the LR estimator can have some undesirable properties due to its sensitivity to sample sizes in terms of variance and bias (Csilléry *et al.*, 2006), and also when faced with highly polymorphic loci or high relatedness values (Blouin, 2003). Our small sample size for *C. polykomos* and its consequences may be reflected in the LR estimator results. All estimators showed high correlation coefficients with almost no differences between them (similar explicatory power) for the two species (Table 4.5). Therefore, we chose the Wang's estimator to assess the relatedness value between every possible pair of individuals because moreover it corrects for sample size bias. The combination of ML-RELATE and MICROCHECKER concerning the presence of null alleles, and of GENEPOP, after the Bonferroni and FDRBH corrections – for deviations from HWE or LD associations – supported the exclusion of the loci mentioned in the Materials and Methods (section "3.3.1.1. Standard genotyping procedures"). The results of the analyses conducted on the reduced datasets did not differ significantly from the full datasets (data not shown). Hence, final analyses were conducted with the full datasets, i.e. 10 loci for *P. badius* and 11 loci for *C. polykomos*. The minimum number of successfully genotyped loci per sample was 60% (6/10 loci) for *P. badius* and 91% (10/11 loci) for *C. polykomos*. The criteria differed between the two species in order to use the maximum number of samples for each, and avoiding excluding valuable information.

Table 4.4 - Results from RELATED (Pew *et al.*, 2015) for the Pearson correlation coefficients between the observed and expected values for each relatedness estimator for *P. badius* and *C. polykomos*.

Estimator	Source	*Piliocolobus badius*	*Colobus polykomos*
LL	Lynch, 1988; Li *et al.*, 1993	0.822	0.811
LR	Lynch and Ritland, 1999	0.815	0.824
QG	Queller and Goodnight, 1989	0.821	0.816
W	Wang, 2002	0.828	0.821

Table 4.5 - Summary statistics and Pearson correlation coefficients between the estimators for *P. badius* on the left and *C. polykomos* on the right. Results obtaied from Coancestry.

	Piliocolobus badius				
	N=406	**LL**	**LR**	**QG**	**W**
	Mean	-0.040	-0.037	-0.036	-0.044
	Var.	0.037	0.013	0.026	0.034
Pearson Corr. Coef.	**LL**	1.000			
	LR	0.761	1.000		
	QG	0.929	0.865	1.000	
	W	0.984	0.777	0.913	1.000

	Colobus polykomos				
	N=28	**LL**	**LR**	**QG**	**W**
	Mean	-0.178	-0.192	-0.147	-0.140
	Var.	0.053	0.050	0.012	0.022
Pearson Corr. Coef.	**LL**	1.000			
	LR	0.887	1.000		
	QG	0.923	0.918	1.000	
	W	0.985	0.886	0.880	1.000

4.3 SEX-BIASED DISPERSAL

The negative values reported in the population assignment test for both species indicated that the individuals belong to the sampled populations. *P. badius* (11 males and 16 females) and *C. polykomos* (3 males and 3 females) showed similar results for the sex-biased dispersal analysis, an absence of significant differences between the sexes (Tables 4.6 and 4.7), suggesting that there is no bias in the dispersal behaviour for both species. The mean AIc was negative for the males (*P. badius*: -0.093; *C. polykomos*: -0.092) and positive for females (*P. badius*: 0.064; *C. polykomos*: 0.092) of both species.

Table 4.6 - *P.badius* population assignement and mean corrected assignment indices. Values shown for both sexes together and separated. Results provided by GenAlEx.

	Piliocolobus badius		
	All Alc	**Male Alc**	**Female Alc**
Mean	0.000	-0.093	0.064
SE	0.359	0.528	0.499
CI	0.704	1.035	0.977
	Two-Tailed Prob. for |Z|	**Lower Tailed Prob. for Z**	**Upper Tailed Prob. for Z**
Probability	0.805	0.403	0.597
Significance	ns	ns	ns

Table 4.7 - *C. polykomos* population assignement and mean corrected assignment indices. Values shown for both sexes together and separated. Results provided by GenAlEx.

	Colobus polykomos		
	All Alc	**Male Alc**	**Female Alc**
Mean	0.000	-0.092	0.092
SE	0.186	0.208	0.348
CI	0.364	0.407	0.683
	Two-Tailed Prob. for |Z|	**Lower Tailed Prob. for Z**	**Upper Tailed Prob. for Z**
Probability	0.827	0.414	0.586
Significance	ns	ns	ns

4.4 GENETIC DIVERSITY

Regarding the populations of *P. badius* and *C. polykomos* from TNP, the former displayed higher genetic diversity than the latter (Tables 4.8 and 4.9). The mean number of different alleles across all loci was higher in *P. badius* (9.900, vs. 5.636 in *C. polykomos*), as was the mean number of effective alleles (5.067, vs. 4.381 in *C. polykomos*), but the difference between these two values was lower in *C. polykomos*. The observed, expected and unbiased expected heterozygosities were higher and more similar in *P. badius* (0.800, 0.795 and 0.809, respectively), while they were lower and more different of each other in *C. polykomos* (0.687, 0.740, 0.790, respectively). In terms of the inbreeding coefficient (F_{IS}), *C. polykomos* showed a higher number of loci with positive values, and therefore a higher mean value (0.141) than *P. badius* (0.012). Given the high genetic diversity, these values may have been originated by genotyping errors. The allelic richness was higher in *P. badius* (mean value of 9.788) than in *C. polykomos* (mean value of 5.443) (Figure 4.1). The allelic richness results from FSTAT and HP-RARE were identical for the same sample sizes. Tables 4.8 and 4.9 display the allelic richness output data from FSTAT when removing individuals with missing data (*P. badius*, N=27; *C. polykomos*, N=7), and (Figure 4.1) is based on the results from HP-RARE with the full datasets. The curves in the rarefaction analysis obtained show a typical logarithmic growth: *P. badius* – $y=2.6535\ln(x)+1.2674$, $R^2=0.9963$; *C. polykomos* (TNP) – $y=1.8287\ln(x)+1.7432$, $R^2=0.9988$; *P. temminckii* – $y=0.6505\ln(x)+2.1853$, $R^2=0.9504$; *C. polykomos* (CNP) – $y=0.6695\ln(x)+1.6661$, $R^2=0.9949$.

The comparison between the results for the TNP and the CNP populations revealed clear differences. The mean number of alleles per locus, as well as the mean allelic richness, was much higher in the TNP populations (*P. badius*: 9.900; *C. polykomos*: 5.636) than in the CNP populations (*P. temminckii*: 4.800; *C. polykomos*: 4.455). This pattern was also observed for the heterozygosity indices: observed heterozygosity (TNP - *P. badius*: 0.800; *C. polykomos*: 0.687; CNP - *P. temminckii*: 0.527; *C. polykomos*: 0.496); expected heterozygosity (TNP - *P. badius*: 0.795; *C. polykomos*: 0.740; CNP - *P. temminckii*: 0.563; *C. polykomos*: 0.477). Lastly, the values of F_{IS} were negative for both CNP's populations (*P. temminckii*: -0.036; *C. polykomos*: -0.127) (Tables 4.8, 4.9 and 4.10).

Table 4.8 – Genetic diversity indices for the microsatellite loci of *P. badius*; ASR= Allele size range; N= Number of successfully genotyped samples; Na= No. of different alleles; Ne= No. of effective alleles; Ho= Observed heterozygosity; uHe= Unbiased expected heterozygosity; SE= standard error. F_{IS} and allelic richness (AR; N=27) measured in FSTAT.

Piliocolobus badius								
Locus	**ASR**	**N**	**Na**	**Ne**	**Ho**	**uHe**	**F_{IS}**	**AR**
D1s1665	171-219	29	11	3.351	0.724	0.714	-0.015	10.583
D4s2408	287-343	28	9	5.917	0.893	0.846	-0.056	8.999
D13s321	153-189	27	10	4.585	0.815	0.797	-0.023	10.000
D6s474	119-187	29	10	4.509	0.759	0.792	0.043	9.8555
D2s1326	195-267	28	13	4.404	0.714	0.787	0.094	12.786
Fesps	150-186	28	8	4.261	0.750	0.779	0.038	7.927
D11s2002	134-202	29	10	5.986	0.759	0.848	0.107	9.879
D2s442	282-338	28	12	6.788	0.857	0.868	0.013	11.927
D6s503	257-281	29	7	5.191	0.828	0.822	-0.007	6.996
D6s1056	238-282	29	9	5.682	0.897	0.838	-0.071	8.924
Mean			9.900	5.067	0.800	0.809	0.012	9.788
SE			1.700	0.975	0.064	0.043	0.056	1.640

Table 4.9 – Genetic diversity indices for the microsatellite loci of *C. polykomos*; ASR= Allele size range; N= Number of successfully genotyped samples; Na= No. of different alleles; Ne= No. of effective alleles; Ho= Observed heterozygosity; uHe= Unbiased expected heterozygosity; SE= standard error. F_{IS} and allelic richness (AR; N=7) measured in FSTAT.

Colobus polykomos								
Locus	**ASR**	**N**	**Na**	**Ne**	**Ho**	**uHe**	**F_{IS}**	**AR**
D1s548	196-216	8	6	5.120	0.625	0.858	0.286	5.858
D1s1665	176-212	8	5	3.200	0.750	0.733	-0.024	4.742
D4s2408	259-283	8	4	3.282	0.625	0.742	0.167	3.875
D13s321	162-174	7	4	2.130	0.429	0.571	0.265	4.000
D6s474	126-154	8	7	5.818	1.000	0.883	-0.143	6.733
D2s1326	211-227	8	5	3.879	0.375	0.792	0.543	4.867
Fesps	138-154	8	4	3.657	0.875	0.775	-0.140	3.992
D11s2002	146-202	8	8	5.818	0.500	0.883	0.451	7.483
D2s442	274-314	8	7	5.565	1.000	0.875	-0.155	6.625
D6s503	293-337	8	8	7.111	0.875	0.917	0.049	7.717
D6s1056	257-285	8	4	2.612	0.500	0.658	0.253	3.983
Mean			5.636	4.381	0.687	0.790	0.141	5.443
SE			1.553	1.511	0.216	0.103	0.232	1.420

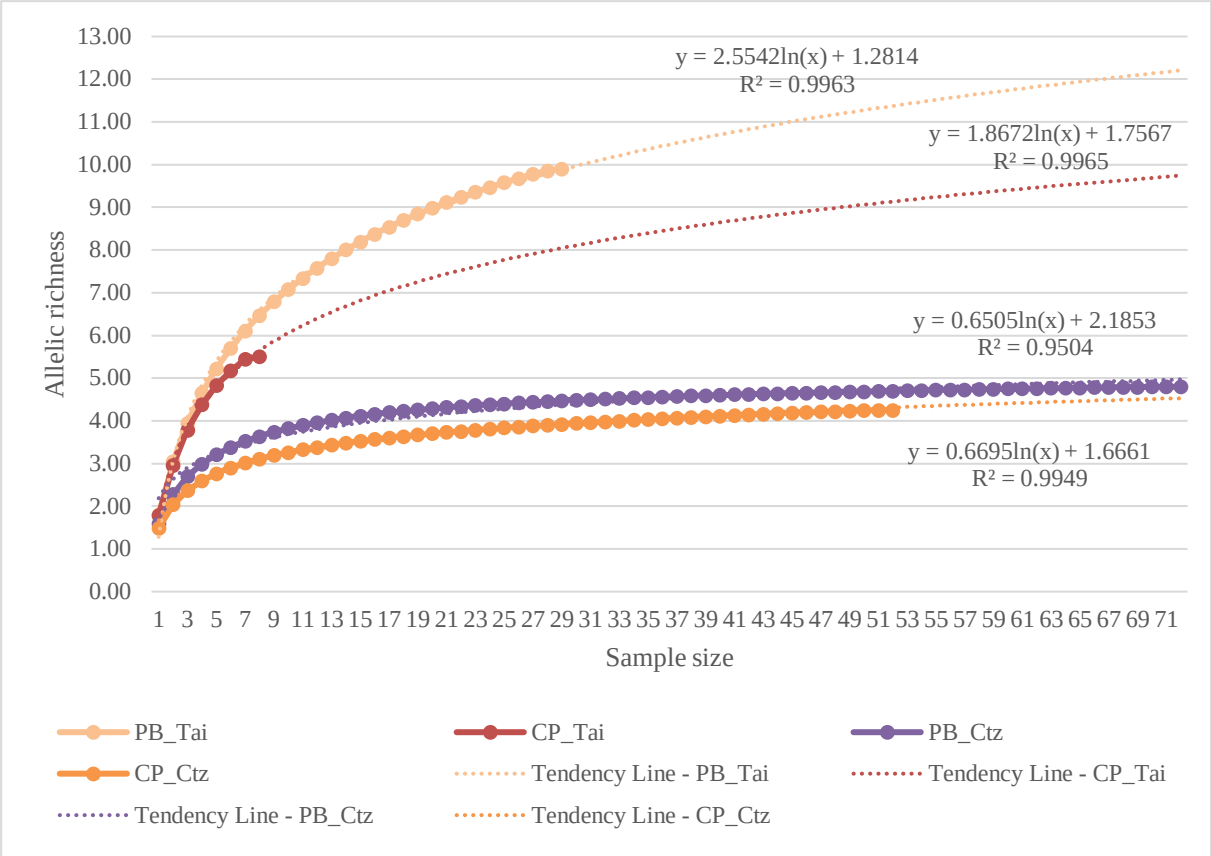


Figure 4.1 - Allelic richness estimated cumulatively by fitting the rarefaction value of each individual. The values were extrapolated to the maximum number of samples (71) through logarithmic tendency lines. PB=*P.badius*; CP=*C.polykomos*; Tai=TNP; Ctz=CNP.

Table 4.10 - Diversity indices for the microsatellite loci of *P. temminckii* and *C. polykomos* from CNP. Adapted from Minhós *et al.* (2013b).

	Piliocolobus temminckii						***Colobus polykomos***					
Locus	**ASR**	**Na**	**Ho**	**He**	**F_{IS}**	**AR**	**ASR**	**Na**	**Ho**	**He**	**F_{IS}**	**AR**
D1s548							200-216	5	0.669	0.622	-0.197	2.495
D1s1665	187-203	5	0.382	0.555	0.225	1.612	164-176	4	0.589	0.537	-0.182	2.265
D4s2408	275-311	6	0.398	0.464	0.046	1.581	259-283	5	0.650	0.560	-0.261	2.103
D13s321	165-169	2	0.289	0.321	0.008	1.307	158-182	6	0.730	0.651	-0.224	2.586
D6s474	131-183	4	0.689	0.576	-0.281	1.571	122-134	4	0.638	0.564	-0.212	2.465
D2s1326	215-239	7	0.836	0.736	-0.247	1.750	215-239	5	0.580	0.590	-0.062	2.381
Fesps	146-162	5	0.540	0.561	-0.056	1.613	138-158	4	0.057	0.056	-0.098	1.122
D11s2002	158-182	4	0.544	0.503	-0.171	1.484	174-186	4	0.641	0.494	-0.381	2.215
D2s442	294-338	7	0.586	0.777	0.107	1.748	293-329	4	0.135	0.162	0.058	1.339
D6s503	269-277	3	0.327	0.453	0.138	1.476	265-293	3	0.414	0.380	-0.180	1.984
D6s1056	230-266	5	0.678	0.688	-0.130	1.745	247-267	5	0.356	0.630	0.340	2.376
Mean		4.800	0.527	0.563	-0.036	1.589		4.455	0.496	0.477	-0.127	2.121
SE		1.536	0.168	0.133	0.161	0.134		0.782	0.216	0.189	0.183	0.454

4.5 POPULATION STRUCTURE

All analysis conducted to infer the structure of the populations of *P. badius* and *C. polykomos* reached the same conclusion. All individuals from each species belong to one single unstructured genetic cluster with identical admixture proportions and genetic composition. Figures 4.2 and 4.3 a reveal the STRUCTURE 2.3.4 results for two (K=2) and three (K=3) genetic clusters for each species. Since we conducted five runs for each hypothesis, we randomly selected one of the runs as representative. The runs conducted for other values of K are not shown due to their lack of relevance. The Evanno's ΔK method did not suggest a lack of structure at first instance, suggesting that the population structure of *P. badius* would be best explained by K=3 (5.282; Figure 4.4), and that of *C. polykomos* K=2 (1.504; Figure 4.4). However, these results were weakly supported, and the analysis of the L(K) results supported the inference of a lack of population structure: for *P. badius*, L(1) ranged between -1117.7 and -1118.7 in the five replicate runs, while L(K) was considerable lower for other values of K (Figure 4.4); for *C. polykomos*, the pattern was not as clear and L(K) was greater for K=2, but L(K=1) was within the standard deviation of L(K=2) (Figure 4.4). In fact, the difference in L(K) for the different values of K tested was very small, not allowing to confidently decide for a most supported K value (e.g. $-294.7 \leq L(1) \geq -295.4$; $-292.4 \leq L(2) \geq -296.2$; $-294.2 \leq L(5) \geq -297.6$). Considering such results, together with the sample size for *C. polykomos* and previous knowledge, we selected K=1 as the most probable to describe the population structure of the species, following Pritchard *et al*. (2000a) recommendation: "we may not always be able to know the TRUE value of K, but we should aim for the smallest value of K that captures the major structure in the data". The FCAs supported our interpretation of the STRUCTURE results for a single genetic cluster in each species. The first axes of the FCAs explained 19.67% of the variation in *P. badius* and 46.69% of the variation in *C. polykomos* (Figure 4.5).

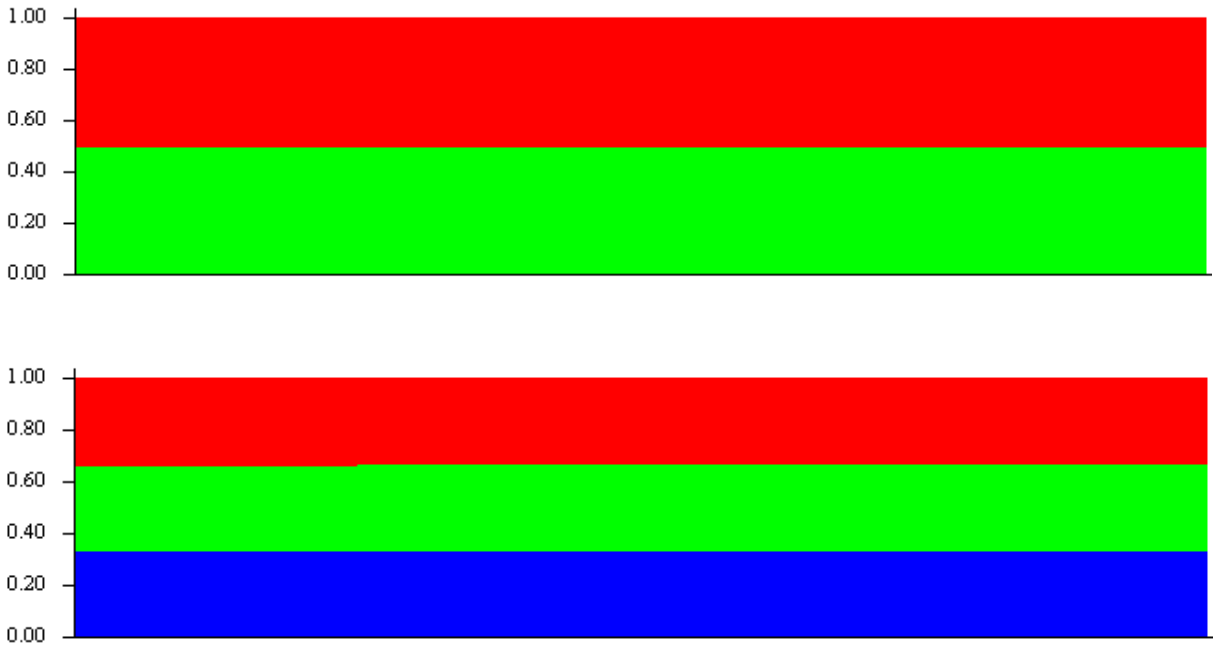


Figure 4.2 - Population genetic clustering of *P.badius* (top - K=2; bottom - K=3) using STRUCTURE. Each column is representative of a single individual.

Figure 4.3 – Population genetic clustering of *C. polykomos* (top - K=2; bottom - K=3) using STRUCTURE. Each column is representative of a single individual.

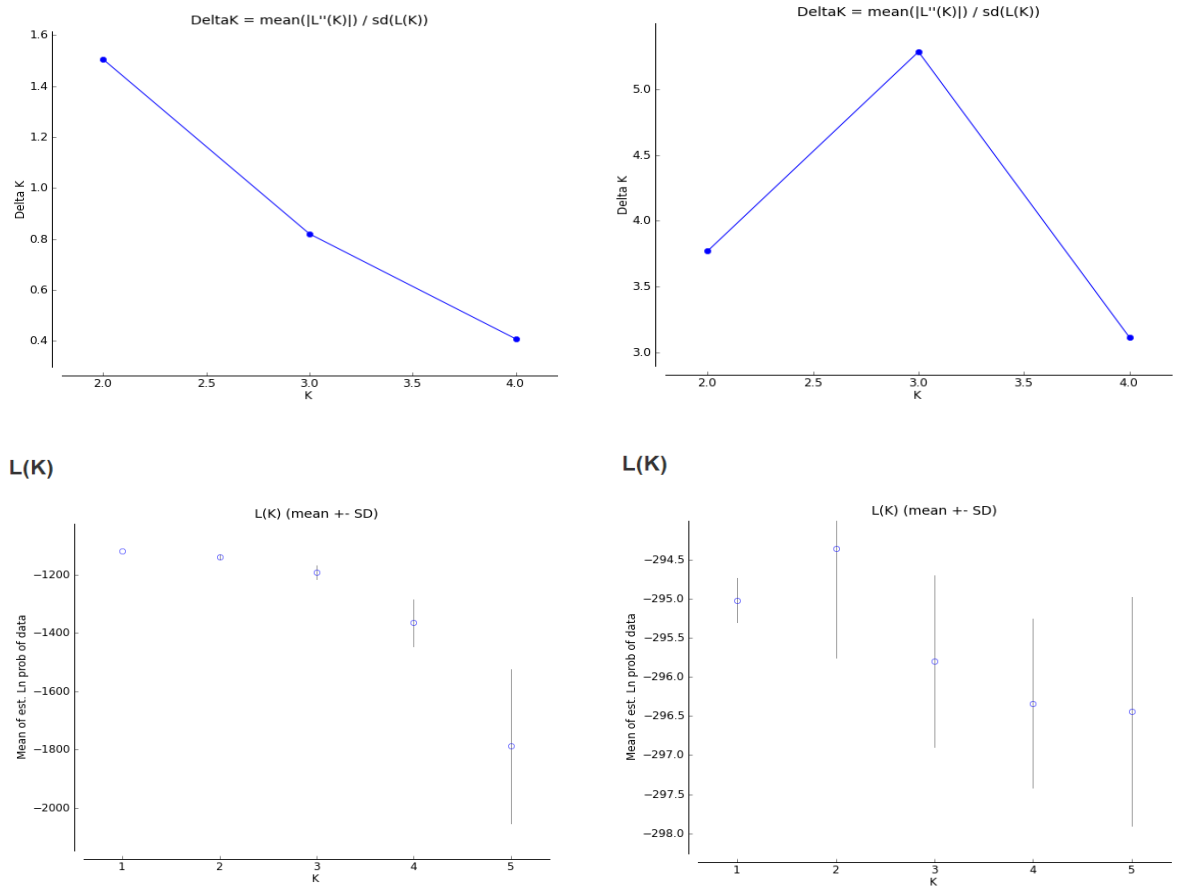


Figure 4.4 – Top: resulting most fitting K by Evanno's ΔK method for *P. badius* (top-left) and *C. polykomos* (top-right). Bottom: L(K) analysis for *P. badius* (bottom-left) and *C. polykomos* (bottom-right). Results obtained from STRUCTURE HARVESTER online. Numeric results in Table AT5.

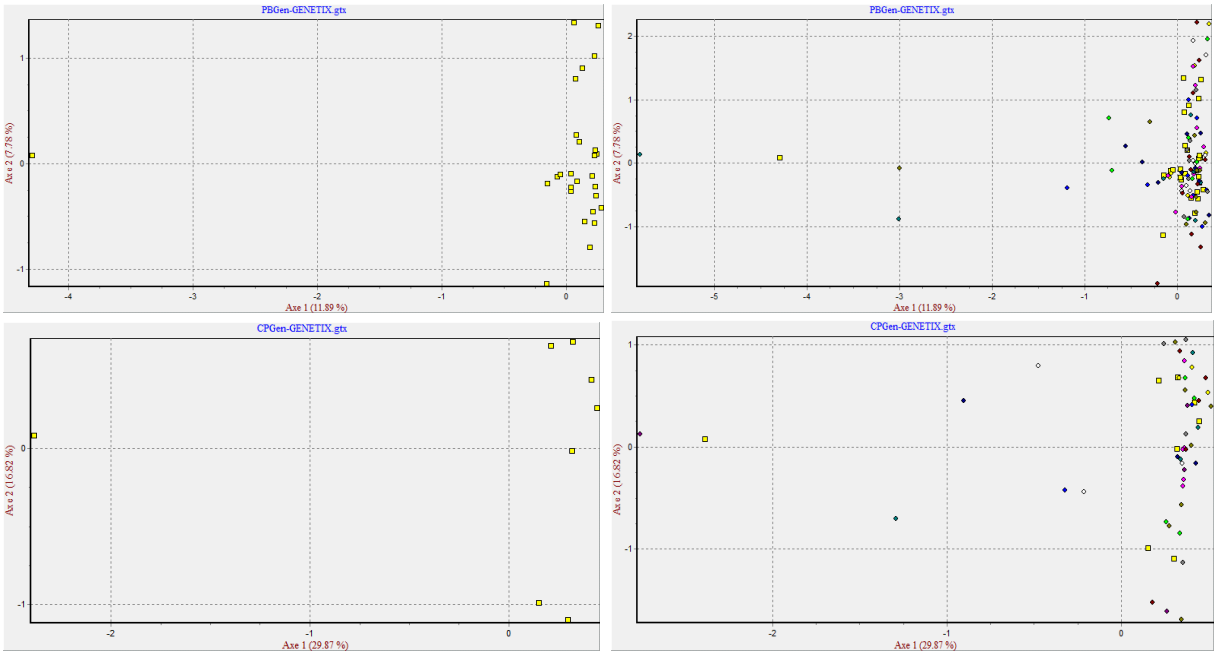


Figure 4.5 – Two-dimensional factorial correspondence analysis of *P. badius* individuals (top-left) and overlapped genotypes (top-right), and *C. polykomos* individuals (bottom-left) with the overlaping genotypes (bottom-right) produced in GENETIX.

4.6 DEMOGRAPHIC HISTORY

The Wilcoxon sign-rank test for heterozygosity excess did not show any significant departure from mutation-drift equilibrium in *P. badius*, for any of the three mutation models tested (Table 4.11), while a significant heterozygosity deficiency was detected under the SMM (p-value=0.02686). On the other hand, in *C. polykomos*, assuming either the IAM (p-value=0.00085) or the TPM (p-value=0.00525), but not with the SMM (p-value=0.05493), there was a significant signal of a demographic bottleneck (Table 4.12).

Table 4.11 - Bottleneck results for *P. badius*. TPM with 30% IAM and 70%SMM. 10000 replications. N=sample size; NAl=Number of alleles; ObsHe=Observed Heterozygosity; IAM=Infinite Allele Model; TPM=Two-Phase Model; SMM=Stepwise Mutation Model; Heq=Expected Heterozygosity; Prob.=Probability (p-value).

Piliocolobus badius									
Locus	**N**	**NAl**	**ObsHe**	**IAM**		**TPM**		**SMM**	
				Heq	**Prob.**	**Heq**	**Prob.**	**Heq**	**Prob.**
D1s1665	58	11	0.714	0.801	0.1021	0.843	0.0148	0.872	0.0005
D4s2408	56	9	0.846	0.750	0.0709	0.800	0.1952	0.838	0.5004
D13s321	54	10	0.797	0.781	0.4879	0.827	0.2078	0.859	0.0408
D6s474	58	10	0.792	0.776	0.4933	0.824	0.1984	0.856	0.0357

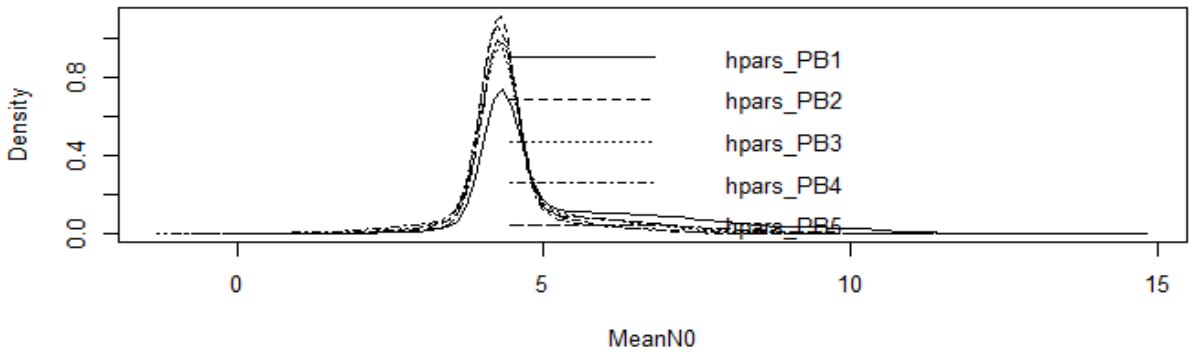
D2s1326	56	13	0.787	0.842	0.1259	0.875	0.0170	0.896	0.0012
Fesps	56	8	0.779	0.716	0.3011	0.772	0.4572	0.813	0.1707
D11s2002	58	10	0.848	0.776	0.1437	0.823	0.3578	0.856	0.3195
D2s442	58	13	0.895	0.839	0.0844	0.874	0.2671	0.896	0.4298
D6s503	56	12	0.868	0.823	0.2285	0.860	0.5102	0.885	0.1870
D6s1056	58	7	0.822	0.671	0.0228	0.732	0.0664	0.783	0.2234
D1s1665	58	9	0.838	0.749	0.0973	0.797	0.2460	0.836	0.4566
Sign Test Prob.				0.12608		0.52209		0.03544	
Wilcoxon Test Prob.		**1Tail H Def**		0.91260		0.61768		0.02686	
		1Tail H Exc		0.10303		0.41553		0.98950	
		2Tails H Exc Def		0.20605		0.83105		0.05371	

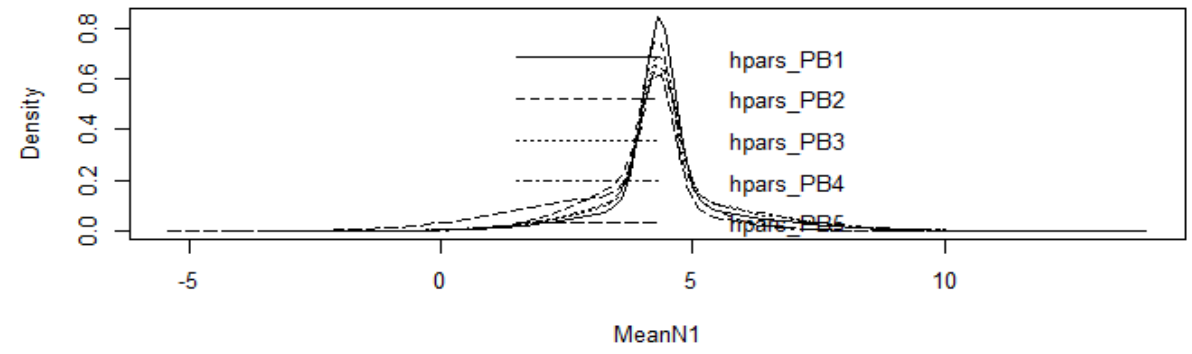
Table 4.12 - Bottleneck results for *C. polykomos*. TPM with 30% IAM and 70%SMM. 10000 replications. . N=sample size; NAl=Number of alleles; ObsHe=Observed Heterozygosity; IAM=Infinite Allele Model; TPM=Two-Phase Model; SMM=Stepwise Mutation Model; Heq=Expected Heterozygosity; Prob.=Probability (p-value).

Colobus polykomos									
Locus	**N**	**NAl**	**ObsHe**	**IAM**		**TPM**		**SMM**	
				Heq	**Prob.**	**Heq**	**Prob.**	**Heq**	**Prob.**
D1s548	16	6	0.858	0.775	0.0528	0.797	0.0918	0.815	0.1557
D1s1665	16	5	0.733	0.709	0.5002	0.736	0.4251	0.759	0.2902
D4s2408	16	4	0.742	0.616	0.1303	0.650	0.1910	0.678	0.2810
D13s321	14	4	0.571	0.638	0.2602	0.666	0.1672	0.693	0.0946
D6s474	16	7	0.883	0.825	0.0969	0.843	0.1658	0.855	0.2590
D2s1326	16	5	0.792	0.705	0.1799	0.734	0.2694	0.760	0.4027
Fesps	16	4	0.775	0.615	0.0338	0.649	0.0494	0.678	0.0857
D11s2002	16	8	0.883	0.864	0.4097	0.876	0.5463	0.886	0.4347
D2s442	16	7	0.875	0.825	0.1406	0.843	0.2406	0.855	0.3473
D6s503	16	8	0.917	0.864	0.0294	0.875	0.0533	0.885	0.0891
D6s1056	16	4	0.658	0.615	0.4158	0.647	0.4894	0.680	0.3438
D1s1665	16	4	0.725	0.613	0.1785	0.650	0.2639	0.679	0.3869
Sign Test Prob.				0.02252		0.05485		0.42049	
Wilcoxon Test Prob.		**1Tail H Def**		0.99939		0.99597		0.95386	
		1Tail H Exc		0.00085		0.00525		0.05493	
		2Tails H Exc Def		0.00171		0.01050		0.10986	

The five independent scenarios we conducted on MSVAR 1.3 revealed an increase in the median of the population size of *P. badius* (overall N_0 of 22716, ranging between 18505 and 37860; overall of N_1 16655, ranging between 11814 and 23443; Table 4.13) and, on the contrary, a decrease in the median of *C. polykomos*' population size (overall N_0 of 5521, ranging between 2863 and 9072; overall N_1 of 13284, ranging between 9444 and 15183; Table 4.14). However, the order of magnitude of both population sizes remained the same. The Brooks, Gelman and Rubin Convergence Diagnostic test (Gelman and Rubin, 1992; Brooks and Gelman, 1998) used to obtain a multivariate potential scale reduction factor confirmed the convergence of the runs as it was, close to 1 (*P. badius*: 1.079; *C.*

polykomos: 1.067), as were the estimates of the corrected scale reduction factor for all parameters (*P. badius*: < 1.156; *C. polykomos*: < 1.097). However, some 0.975 quantiles showed values indicative of absence of convergence, over 1.20 (*P. badius*: MeanN_0=1.376; *C. polykomos*: MeanN_0=1.231) (Figures AF 1, AF 2, AF 3 and AF 4). For both *P. badius* and *C. polykomos*, the posterior distributions of N_0 and N_1 (Figures 4.6 and 4.7) were found to overlap consistently across all scenarios, a result also mimicked by the 95% highest posterior density intervals (*P. badius*: N_0=716–4.88*10^7, N_1=12–1.86*10^7; *C. polykomos*: N_0=9–1.81*10^7, N_1=26–4.36*10^6). The posterior distributions of time (T) for both species displayed a wide range of limits for the moment since the occurrence of a demographic change (Tables 4.13 and 4.14). For *P. badius*, the 95% highest posterior density intervals of T were overall coherent in the magnitude of their lower and upper limits (Table 4.13). With the exception of scenario 1 (median at 5903 years ago), the median for the remaining scenarios dated the occurrence of a demographic change between 1,477,760 and 2,500,789 years ago. Visually, the graphic of mean T showed an unusual double peak pattern (Figure 4.6). For *C. polykomos*, the 95% highest posterior density intervals of T were more variable in the magnitude of their upper limits, ranging from 1.18*10^5 to 1.13*10^9 (Table 4.14). In contrast with *P. badius*, the median values across scenarios were much lower, oscillating between 2,859 and 17,618 years ago. The latter is relatively far from the overall value obtained for the timing of the demographic change (6065 years ago), but in general the event is dated as relatively recent. The double peak pattern was again visible in the mean T graphic, but this time without any evident discrepancy between runs (Figure 4.7). We consider that the consistent overlap between N_0 and N_1 curves, supported by the overlap of the respective 95% highest posterior density intervals, and the unusual double peak patterns of time suggests the absence of any demographic change for both species. This inference is especially relevant for *P. badius*, since MSVAR 1.3 dated the change for a considerably ancient period.





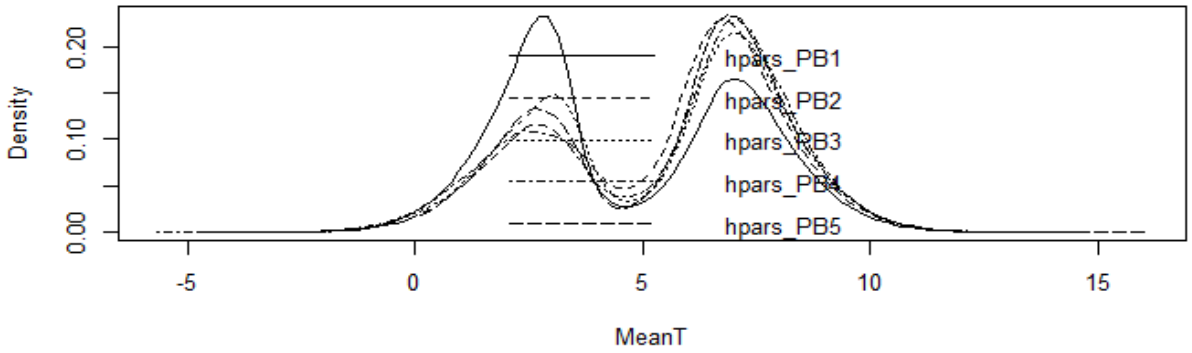


Figure 4.6 - Posterior distributions of MSVAR 1.3 parameters' means in a logarithmic scale for *P.badius*: current effective population size (N0; top), ancestral population size (N1; middle) and the time (T) since the occurrence of the demographic change (bottom). The five scenarios/runs are presented and differentiated by the type of line, as reported by the inherent subtitle.

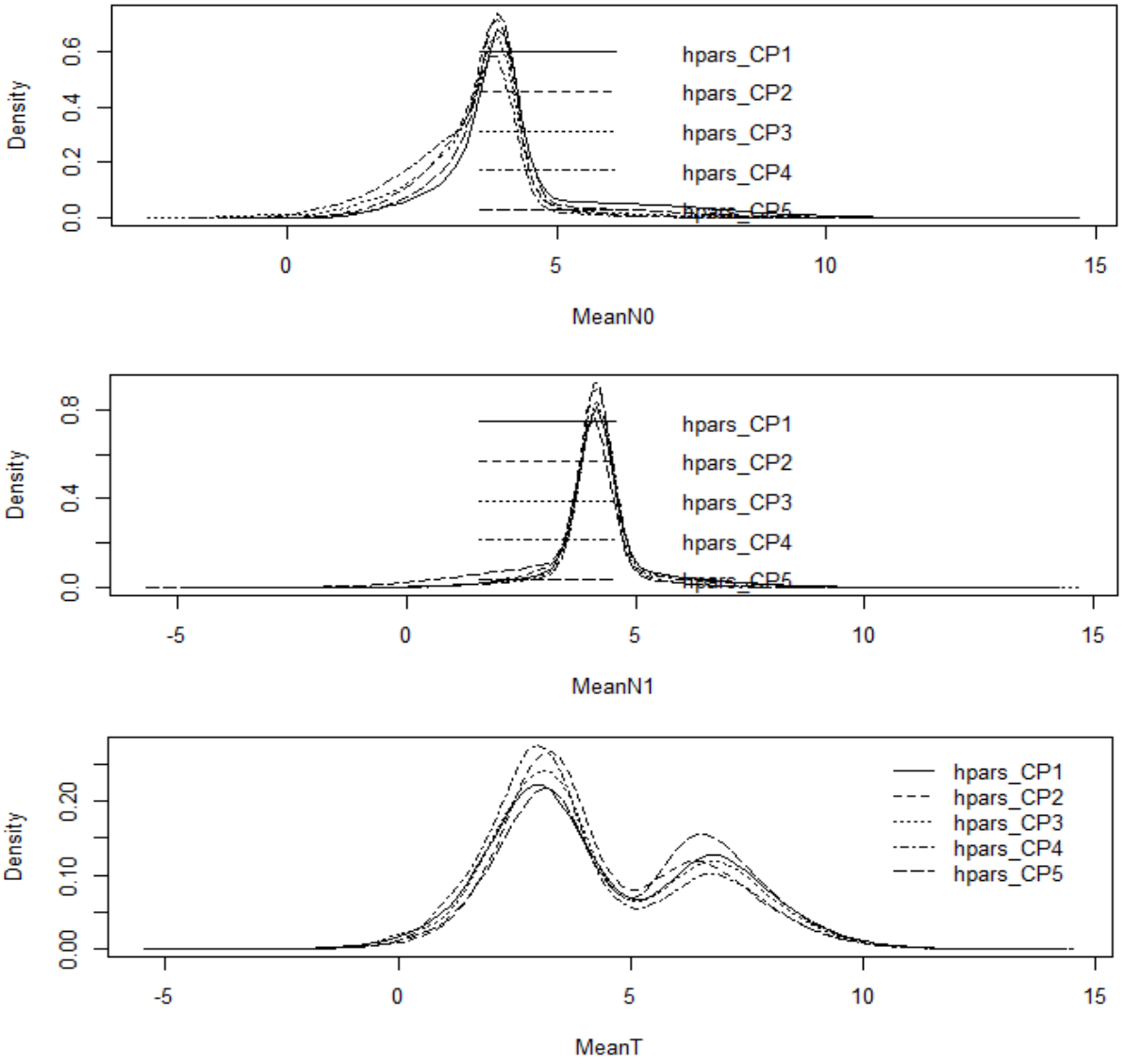


Figure 4.7 - Posterior distributions of MSVAR 1.3 parameters' means in a logarithmic scale for *C. polykomos*: current effective population size (N0; top), ancestral population size (N1; middle) and the time (T) since the occurrence of the demographic change (bottom). The five scenarios/runs are presented and differentiated by the type of line, as reported by the inherent subtitle.

Table 4.13 - Posterior distributions of the current population size (N0), ancestral population size (N1) and the time since the demographic change (T), per scenario and overall, for *P. badius*. Data collected from MSVAR 1.3.

		Piliocolobus badius					
		Sce.1	**Sce.2**	**Sce.3**	**Sce.4**	**Sce.5**	**Overall**
N_0	**Mean**	219385	24040	35089	20378	51125	45396
	Median	37860	20030	22040	18505	23790	22716
	HPD95%	$2768 – 3.32*10^9$	$1442 – 1.43*10^6$	$1042 – 1.41*10^7$	$229 – 1.82*10^6$	$2089 – 4.53*10^7$	$716 – 4.88*10^7$
N_1	**Mean**	28523	12176	31276	31089	3795	16655
	Median	23075	16468	23352	23443	11814	19436
	HPD95%	$76 – 2.90*10^7$	$49 – 1.07*10^6$	$44 – 5.63*10^7$	$33 – 6.72*10^7$	$1 – 1.22*10^6$	$12 – 1.86*10^7$
T	**Mean**	46243	300219	229094	339685	326134	203889
	Median	5903	1571310	1477760	2500789	2258234	1318357
	HPD95%	$2 – 2.48*10^9$	$4 – 3.30*10^9$	$3 – 3.97*10^9$	$2 – 4.82*10^9$	$4 – 4.52*10^9$	$3 – 3.84*10^9$

Table 4.14 - Posterior distributions of the current population size (N0), ancestral population size (N1) and the time since the demographic change (T), per scenario and overall, for *C. polykomos*. Data collected from MSVAR 1.3.

		Colobus polykomos					
		Sce.1	**Sce.2**	**Sce.3**	**Sce.4**	**Sce.5**	**Overall**
N0	**Mean**	18903	3705	3082	1712	7825	4923
	Median	9072	4962	4618	2863	6986	5521
	HPD95%	$43 – 9.24*10^7$	$35 – 1.01*10^5$	$6 – 3.90*10^5$	$6 – 5.07*10^4$	$38 – 4.10*10^6$	$9 – 1.81*10^6$
N1	**Mean**	18402	10877	18815	18734	4332	12509
	Median	14344	12659	15183	15081	9444	13284
	HPD95%	$81 – 1.31*10^7$	$113 – 3.31*10^5$	$93 – 1.09*10^7$	$168 – 1.07*10^7$	$1 – 4.33*10^5$	$26 – 4.36*10^6$
T	**Mean**	27459	23533	22899	12312	52546	24901
	Median	7058	6445	5481	2859	17618	6065
	HPD95%	$4 – 1.11*10^9$	$9 – 4.90*10^8$	$5 – 1.13*10^9$	$4 – 4.93*10^8$	$7 – 1.18*10^5$	$5 – 7.86*10^8$

The preliminary runs for *P. badius* in VAREFF indicated a decrease between ancestral (Theta2=57.009) and intermediate (Theta1=51.430) Ne, and an abrupt decrease towards the present values (Theta0=13.213). Consequently, both imbalance indices were positive (I1=1.359; I2=1.462). For the mutation rate of 0.001, the current values of Ne should be between 3303 and 14252. Of the reported indices, we will rely essentially in the median, because the arithmetic and harmonic means are outperformed by the median robustness in terms of sensitivity to the number of markers used (Nikolic and Chevalet, 2014). The posterior distributions of Ne in *P. badius* reinforced what the preliminary analysis had already indicated: an ancient bottleneck starting around the 30,000th generation (150,000 years ago, assuming a generation time previously estimated of 5 years; T(30000)=71836.6; T(28500)=36830.5) that halved the population size, and a severe recent bottleneck in the last 20 generations (T(20)=26954.8; T(0)=46.5), that took the current Ne to a range between 14.0 and 106.6 Table 4.15. All representations of the posterior distributions of Ne resemble this four-stage scenario: an ancient phase of steady decrease (T(50000)=114769.9), followed by a first bottleneck that caused a gradual decrease until a second stationary phase (with a Ne of 28000 to 30000), and then a very recent bottleneck (Figure 4.8). It must be noticed that the 95% highest probability density intervals overlapped continuously. The realized acceptation rate of the *P. badius*' simulation was 0.254.

Table 4.15 – Posterior distributions of *P. badius* effective population size at different times. Tg=time in generations before present; Ty=time in years before present; AMean=arithmetic mean; HMean=harmonic mean; HPD95%= 95% highest probability density interval; SD=standard deviation. Data collected from VAREFF.

Piliocolobus badius							
Tg	**Ty**	**AMean**	**HMean**	**Mode**	**Median**	**HPD95%**	**SD**
0	0	57.9	34.7	42.2	46.5	14. 0 – 106.6	444.9
20	100	21781.3	368.4	89.2	26594.0	75.6 – 34810.9	12800.5
40	200	28472.1	8851.5	26788.0	28052.4	22900.8 – 35762.2	5639.4
60	300	28826.8	26889.0	26734.4	28124.8	23203.6 – 35865.4	4775.9
80	400	28839.5	27941.1	26756.4	28126.0	23209.0 – 35866.1	4765.0
100	500	28842.8	28213.2	26756.4	28126.1	23210.0 – 35866.1	4756.6
500	2500	28842.8	28213.2	26756.4	28126.1	23210.0 – 35866.1	4756.6
5000	25000	28842.8	28213.2	26756.4	28126.1	23210.0 – 35866.1	4756.6
10000	50000	28940.6	28256.6	26715.1	28136.0	23224.3 – 36054.1	5160.4
15000	75000	30548.8	28905.9	26986.9	28356.5	23472.3 – 42133.7	10440.5
23000	115000	47931.9	35861.2	28991.8	30731.5	25103.0 – 112823.7	31715.1
28500	142500	66570.6	45299.2	30311.0	36830.5	26567.7 – 132694.0	40798.5
29000	145000	68115.5	46226.1	30313.1	40506.4	26660.8 – 134243.9	41229.1
29500	147500	69940.3	47321.4	30376.1	53222.3	26852.4 – 136068.9	41782.2
30000	150000	71282.3	48161.8	30353.4	71836.6	26991.5 – 137350.3	42138.3
50000	250000	116657.2	87461.4	108397.2	114769.9	32317.2 – 206050.4	51795.5

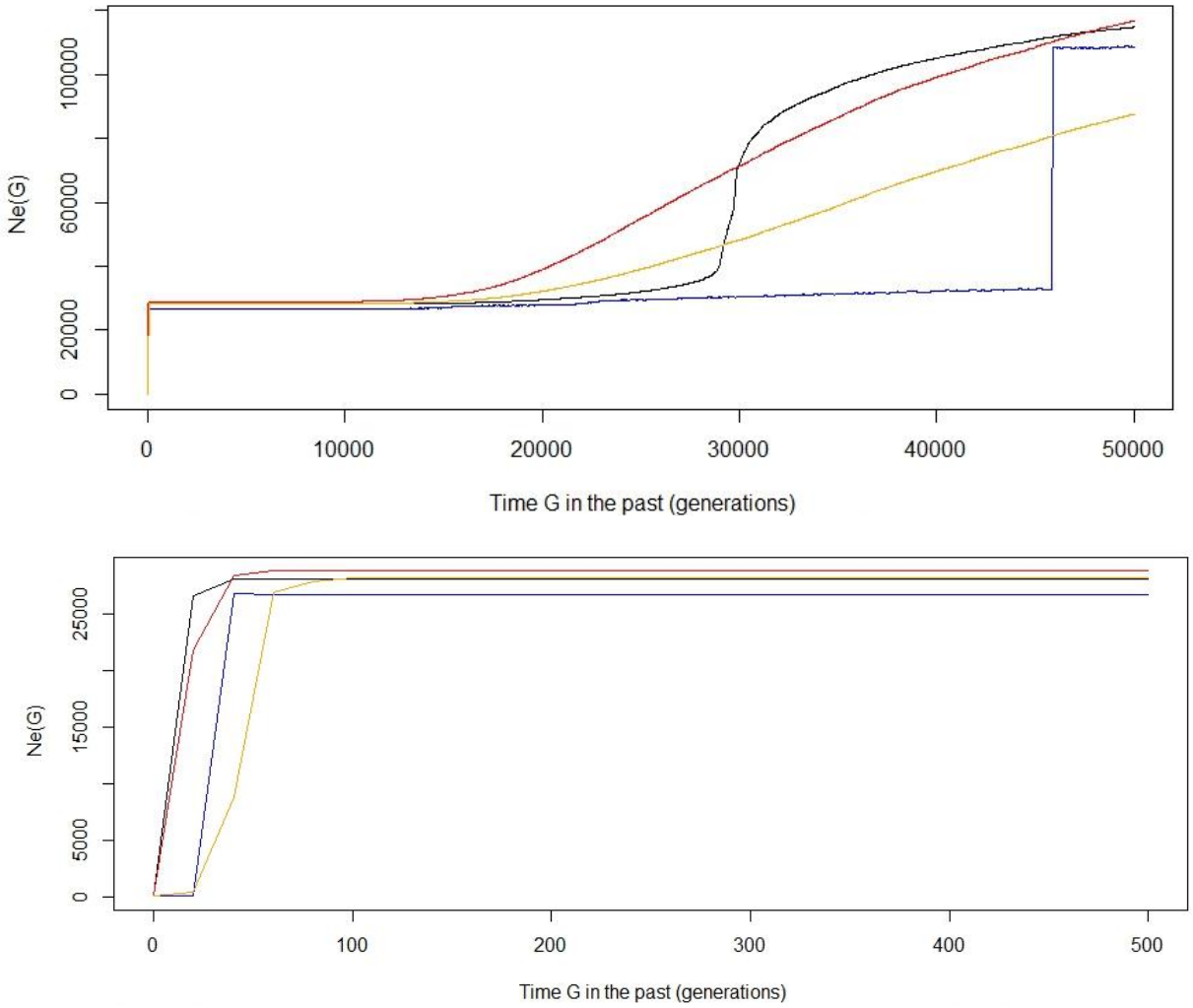


Figure 4.8 – Variation of *P. badius*' Ne in a normal scale through time (T) in generations. Top: Time extended until the 50000th generation to visualize the two detected bottlenecks. Bottom: Time extended until the 500th generation to focus the abrupt sudden decrease. A: red=mean of Ne, blue=mode of Ne, black=median of Ne, orange=harmonic mean of Ne. Plots produced in VAREFF.

For *C. polykomos*, there was a small increase between the ancestral (Theta2=48.167) and the intermediate (Theta1=51.329) Ne, followed by a drastic decline to the current size (Theta0=10.819). The obtained imbalance indices reflected this pattern (I1=1.557; I2=1.493) but there was, however, no support for an intermediate or ancient decline in the runs. For a mutation rate of 0.001, the values of Ne ranged between 3303 and 14252. By analysing the posterior distributions of Ne, we inferred a historically stable population that underwent a recent episode of rapid decline starting 40 generations ago (Table 4.16; 200 years ago; T(40)=32325.6, T(0)=25.2). A closer look at the 95% highest probability density intervals and Figure 4.9 suggests that the decline started earlier, due to the gradual decrease of the HPD95% lower limits (T(10000)=279850.2; T(5000)=35477.8; T(500)=19033.7; T(40)=1492.4). According to these results, the current Ne of *C. polykomos* in the TNP lies between 9.4 and 48.0. Our focus in the lower limits is based on the mentioned methodological bias that supported an ancestral Ne much larger than could be expected, despite the level of the acceptance rate (0.249). We sought a solution for this, and the use of a more conservative mutation rate of 0.0005 corrected this bias without changing the inferred demographic history, but rather the magnitude of the Ne. For a mutation rate of

0.0005, the preliminary results pointed to a Ne ranging from 5409 to 25664. The analysis inferred an ancient and stationary phase with an approximate median of 80,000 individuals (95%HPD, 60,000-130,000; SD, 27,000) and a recent and ongoing bottleneck that rendered a Ne of 1.6 to 9.2 (median: 4.8). The Theta values and the imbalance indices remained the same as before. Without entering in a specific and detailed discussion of these issues, a note should be made about the exploratory variation of the parameters that we conducted (results not shown). The scenarios produced by different combinations of parameters (changing model complexity, mutation rate, among others mentioned by Nikolic and Chevalet, 2014) were divided in two major groups: either agreed with the recent ongoing bottleneck mentioned above (the most frequent result) or were doubtful, considering their deviations from the acceptance rate or their lack of correlation with natural or anthropogenic events known from the literature. Regarding the use of SSM to predict data generated under a more complex model, the authors of VAREFF found a tendency for an overestimation of ancient population size and spurious detection of current bottlenecks. Theoretically, the detection of an ongoing bottleneck depends on the allele frequencies in the last generations, a function of drift instead of mutations, which in turn affect older frequency distributions.

Table 4.16 - Posterior distributions of *C. polykomos* effective population size at different times. Mutation rate of 0.001. Tg=time in generations before present; Ty=time in years before present; AMean=arithmetic mean; HMean=harmonic mean; HPD95%=95% highest probability density interval; SD=standard deviation. Data from VarEff.

Colobus polykomos							
Tg	**Ty**	**AMean**	**HMean**	**Mode**	**Median**	**HPD95%**	**SD**
0	0	34.8	20.3	23.7	25.2	9.4 – 48.0	269.6
20	100	62278.6	101.9	0.0	7923.1	30.8 – 363525.0	214266.5
40	200	132972.7	3606.4	8062.4	32325.6	1492.4 – 538481.2	292932.3
60	300	158978.3	12779.6	22272.0	38459.2	2985.0 – 556757.5	302449.0
80	400	175929.6	18284.6	27840.0	43935.9	4335.7 – 560686.5	304976.9
100	500	189098.1	22992.7	30624.0	48358.0	5595.6 – 567482.0	306433.0
250	1250	235317.6	47166.5	36192.0	85772.4	15079.9 – 577395.0	307186.2
500	2500	259280.3	60260.2	38976.0	185836.7	19033.7 – 587234.2	305777.9
5000	25000	409747.4	172097.0	395220.7	407624.0	35477.8 – 731131.5	286125.6
10000	50000	495435.0	386577.4	400131.2	443474.2	279850.2 – 859554.2	272017.9
15000	75000	509545.7	435197.7	399082.5	449604.2	311987.2 – 874109.5	270574.0
23000	115000	516018.6	451750.5	400608.8	451925.0	319915.5 – 886314.5	276109.4
28500	142500	517308.3	455748.2	400608.8	452630.5	321692.8 – 889211.8	275790.7
29000	145000	517377.9	39421.1	400608.8	452630.5	321832.5 – 889211.8	275751.7
29500	147500	517377.9	39421.1	400608.8	452630.5	321832.5 – 889211.8	275751.7
30000	150000	517414.7	455974.6	400608.8	452644.8	321850.0 – 889211.8	275729.7
50000	250000	518945.6	459669.6	400448.5	453018.2	323868.7 – 892504.7	276757.8

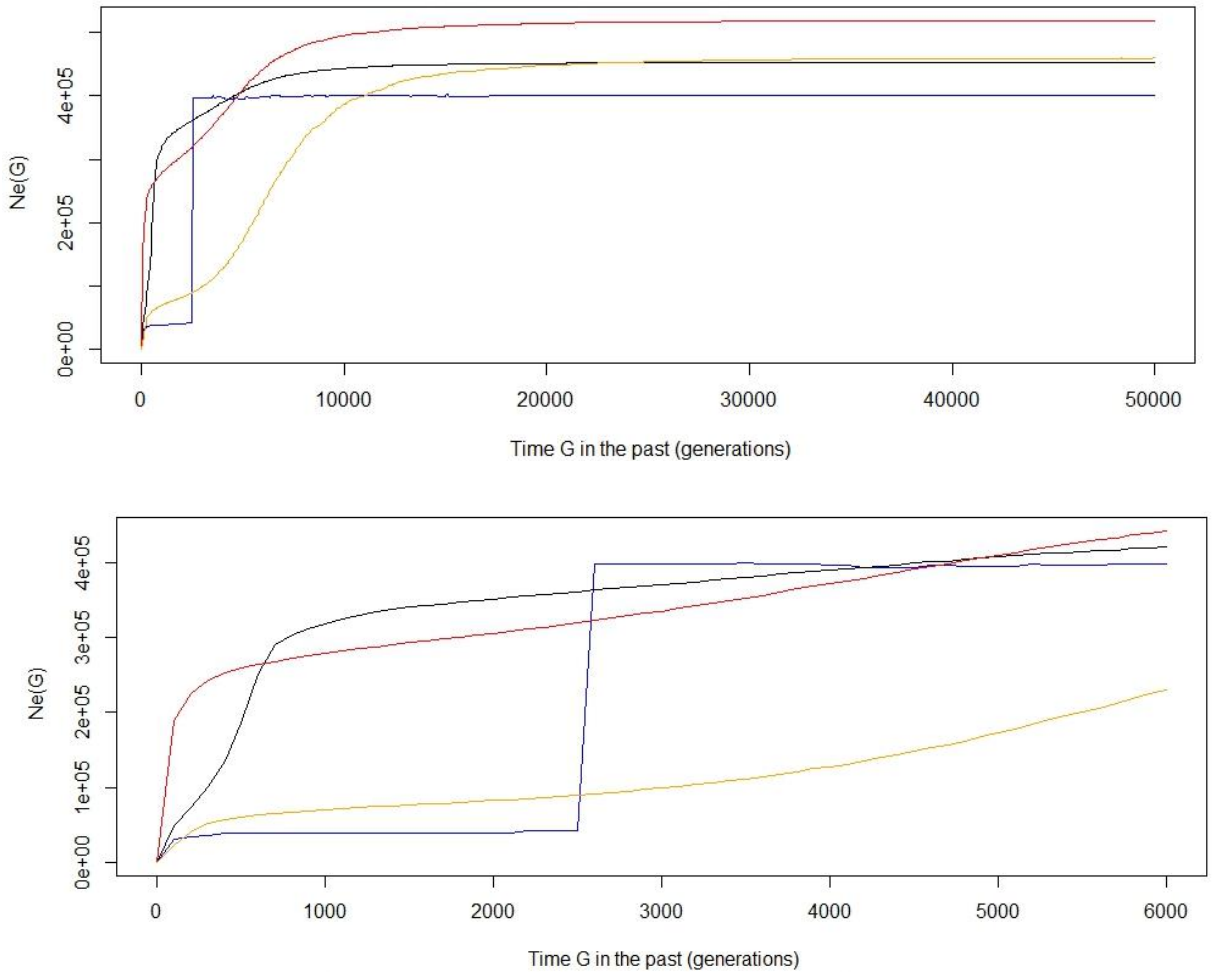


Figure 4.9 – Variation of *C.polykomos*' Ne in a normal scale through time (T) in generations. Top: Time extended until the 50000th generation to visualize long unchanged effective population size. Bottom: Time extended until the 6000th generation to focus the gradual and abrupt sudden decrease. A: red=mean of Ne, blue=mode of Ne, black=median of Ne, orange=harmonic mean of Ne. Plots produced in VAREFF.

5 DISCUSSION

5.1 SEX-BIASED DISPERSAL: THE COLOBINE'S PLASTICITY

Although in the assignments tests for both species we did not find a significant difference in the dispersal pattern between the sexes, the tendency of the results deserves a closer look. Females exhibited a tendency for positive scores and males a tendency for negative scores, suggesting that females might be the philopatric sex and males the dispersing sex. Being the philopatric sex, females showed higher assignment values to the populations. This pattern we observed is in disagreement with previous studies on *P. badius* and in accordance with those in *C. polykomos*.

The efficiency of the applied assignment tests largely depends upon the sample size and the number and polymorphism of the microsatellites analysed (Goudet *et al*., 2002). A joint analysis of all individuals as we conducted, without differentiating the individuals according to their pre- or post--dispersal status (since we lack the necessary information) might be one the factors masking the expected female-biased dispersal in *P. badius*. Relatively limited number of samples and microsatellites used in the study are also potential sources of error.

Red colobus are known to have complex and variable social systems. Based on a patrilineal social organization, females are the main sex promoting dispersal. Our results suggest either the opposing scenario or, more likely, the absence of sex-biased dispersal. Recently, Miyamoto and colleagues (2013) found evidence of an important life history trait in Tana River red colobus (*P. rufomitratus*) that allows them to modify their dispersal system: the size of the social group. In smaller groups the intraspecific competition is softened and, therefore, females are less likely to disperse. However, since the TNP does not contain barriers of any kind, at least to our knowledge, the group size threshold that triggers this behavioural shift may have not been reached. Moreover, the largest *P. badius* groups (with approximately 100 individuals, which is closer to their ecologically limiting group size) were observed at the TNP (Korstjens and Dunbar, 2007). The occurrence of such group size is explained by the high risk of predation that the species experiences from chimpanzees (Korstjens and Dunbar, 2007) and may represent an alternative to the establishment of interspecific associations (Noë and Bshary, 1997; Korstjens *et al*. 2002; McGraw and Bshary, 2002; Buzzard, 2010). Mbora and Meikle (2004) had suggested that the basal area of food trees, which is associated to the protein-fibre ratios, was the most important variable for colobus population density and average group size. Earlier, Sthrusaker and Leland (1979) had suggested that red colobus that inhabit rainforests had social group sizes that were twice the size in populations of semi-arid areas. The same study reported that red colobus males were also seen dispersing, even if in a lesser degree than females. The ability of both sexes to disperse, and consequent reduction in the strength of sex-biased dispersal, could at least partially explain the absence of significant differences between males and females in the results of the assignment tests. Overall, several facts may be in play, and their relative importance is likely to vary among red colobus species and geographic populations. Red colobus exhibit a substantial socioecological plasticity and lack phylogenetical inertia (Chapman and Rothman, 2009). Still, without any clear ecological explanation for the discrepancy between our result and the consensus from the literature about the nature of the bias in the dispersal of the sexes in red colobus, we must acknowledge the pattern we found may be due to the aforementioned methodological constraints and limitations of the assignment analyses. More observational and genetic studies are needed to clarify this issue.

The genus *Colobus* has also a complex dispersal system, in which different species display different behaviours, and even the same species can show distinct patterns. Korstjens *et al*. (2005) reported that *C. polykomos* dispersal in the TNP mainly involved males, with occasional episodes of female dispersal, an observation that opposed previous theoretical suggestions. This dispersal system is now considered to be the general rule in the genus, since it was also verified in *C. vellerosus* (Techroeb *et al*., 2014; Wikberg *et al*., 2014; Sicotte *et al*., 2015) and *C. guereza* (Harris et al., 2009) and it is in agreement with the tendency showed in our results. Another important consideration about TNP's black-and-white colobus population is that home ranges overlap broadly, reducing the costs of dispersal to adjacent groups (Korstjens *et al*., 2005). The same authors found that Taï's *C. polykomos* females are behaviourally different from other black-and-white colobus females, as they participate in intergroup conflicts when important food sources are at risk. As above for *P. badius*, the ability of both sexes to disperse, and the consequent reduction in the strength of sex-biased dispersal, may explain the absence of significant differences between males and females in the results. Further, the *C. polykomos*' dataset shares the same limitations of the *P. badius* dataset, even to a greater extent in what concerns sample size. In terms of social group size, the smaller size compared to red colobus can be understood by the absence of high chimpanzees' predation pressure in *C. polykomos*, and so this life history trait is mainly driven by resting time constraints required for fall-back food digestion (Korstjens and Dunbar *et al*., 2007). To live in a social group means facing a trade-off between energetic costs and benefits. The benefits are intuitive, but the ecological costs of living in a large social group, with increased intragroup food competition, feeding and foraging time, and home range areas are more difficult to weigh at first sight (Janson and van Schaik, 1988; Janson and Goldsmith, 1995). Larger groups are also likely to be more easily detected by humans and other predators.

5.2 GENETIC DIVERSITY: AN INTRA AND INTERSPECIFIC COMPARISON

The genetic diversity analysis in both species supported the seemingly healthy genetic status we hoped to find in the TNP's colobine populations. Although both species showed a high number of alleles, the difference between the number of alleles and the number of effective alleles was smaller in *C. polykomos*. The latter measure is equivalent to the number of equally frequent alleles required to achieve a given degree of genetic diversity. Therefore, we are able to compare populations where the number of alleles and their respective frequency differed considerably. In *P. badius*, we detected a higher percentage of alleles whose frequencies are far from the overall average (i.e. rare alleles), thus with a smaller contribution to the number of effective alleles. The heterozygosity was high in both species, with *C. polykomos* showing a greater difference between expected and observed heterozygosity; concordantly, *C. polykomos*' F_{IS} value was higher. This may due to the possible presence of null alleles in the loci reported by ML-RELATE, or inbreeding and non-random mating. The rarefaction results and the logarithmic tendency lines show that allelic richness is higher in *P. badius*' than in *C. polykomos*, but that neither reached a stationary phase for our highest sample value, suggesting a good genetic status of both species in TNP. Hence, the observed lower genetic diversity in *C. polykomos* may be a consequence of the smaller sample size analysed for this species. Hale *et al*. (2012) found that the ideal minimum sample size for population genetic studies using microsatellites as molecular marker should be comprised between 25 to 30 individuals.

Comparing our findings with those in other red colobus populations (Table 5.1), Allen *et al*. (2012) elaborated a study focusing the genetic diversity and demographic history of the Tana River red colobus

(*P. rufomitratus*) at the Kibale National Park in Western Uganda, using 10 microsatellites. Like TNP, this park is very well preserved, albeit is unfortunately becoming an isolated block of forest (Chapman *et al*., 2013), and shelters one of the remaining large and unthreatened red colobus populations, with an estimated density rounding 17000 individuals (Struhsaker, 2005), and an effective population size between 2500 and 4000 (Allen *et al*.,2012). In terms of demographic history, at least in the last 40000 years the population does not seem to have suffered any significant size changes. The good ecological conditions in the parl seem to favour a healthy red colobus population, and the genetic data supports this idea. In our case, the TNP's colobus populations seem to be even genetically healthier, especially if we consider the differences in sample sizes between the studies: Allen *et al*.'s study sampled 85 *P. rufomitratus* individuals, whereas we used 29 samples for *P. badius* and eight for *C. polykomos*. One possible explanation for observed differences in genetic diversity is the smaller area of the Kibale National Park, with 795 km^2 of rainforest, and the isolation of its red colobus population, while the carrying capacity at TNP should be greater given its area of 5364 km^2. Further, comparing our results with all the available genetic diversity studies using microsatellites in populations of *Piliocolobus* and *Colobus*, the TNP's *P. badius* population appears to be genetically more diverse than any other (Table 5.1). The phylogenetically close Asian colobines, represented in Table 5.1 by *Rhinopithecus bieti* Milne-Edwards, 1897 and *Rhinopithecus roxellana* Milne-Edwards, 1870, also exhibited a lower genetic diversity.

Table 5.1 - Genetic diversity of several colobine species obtained from the present study and from the available literature. All the reported values were provided by studies that used microsatellites as molecular markers. N= Number of successfully genotyped samples; Na= No. of different alleles; Ne= No. of effective alleles; Ho= Observed heterozygosity; He= Expected heterozygosity; F_{IS}=Inbreeding coefficient; AR=Allelic richness; IC=Ivory Coast; GB=Guinea-Bissau;U=Uganda;K=Kenya;T=Tanzania;C=China.

Genetic diversity of colobines									
Species	**Source**	**Site**	**N**	**Na**	**Ne**	**Ho**	**He**	**F_{IS}**	**AR**
P. badius	**This study**	**IC**	**28-29**	**9.90**	**5.07**	**0.80**	**0.80**	**0.01**	**9.79**
P. temminckii	**Minhós *et al*., 2013b**	**GB**	**72**	**5.20**		**0.54**	**0.51**	**-0.05**	**1.63**
P. rufomitratus	Allen *et al*., 2012	U	78-85	7.70	4.32	0.70	0.72	0.04	
P. rufomitratus	Miyamoto *et al*., 2013	U	24-28	5.70	3.93	0.64	0.71	0.10	
		U	31-36	6.40	3.90	0.72	0.71	-0.01	
P. rufomitratus	Mbora and McPeek, 2015	K	72	5.59		0.71	0.70	-0.02	1.68
P. gordonorum	Ruiz-Lopez *et al*., 2016	T	121	4.90	3.21	0.67	0.65	-0.02	4.42
C. polykomos	**This study**	**IC**	**7-8**	**5.64**	**4.38**	**0.69**	**0.79**	**0.14**	**5.44**
C. polykomos	**Minhós *et al*., 2013b**	**GB**	**52**	**4.20**		**0.48**	**0.42**	**-0.14**	**2.06**
R. bieti	Liu *et al*., 2009	C	135	7.50		0.62	0.70	0.12	7.50
R. roxellana	Chang *et al*., 2012	C	202	7.44		0.61	0.67	0.01	5.52

Little is known about the population genetics and genetic diversity of the different species of *Colobus*. Compared with other African and Asian colobus' populations (Table 5.1), the TNP's *C. polykomos* population possesses a genetic diversity within the range found in populations of the different species, with lower values in some of the indices, but higher in others. Given our very small sample size, the genetic diversity of the TNP's *C. polykomos* population may in reality be larger than in most of the studied colobus populations and similar to that of TNP's P. *badius*.

5.3 POPULATION STRUCTURE: IN THE ABSENCE OF BARRIERS

The population genetic structure of a species is one of the crucial aspects that should compose every conservation genetics study, and its knowledge is required to interpret, understand and validate genetic diversity estimates and demographic history inferences. There are many factors promoting (or preventing) genetic partition of populations, from landscape features and barriers to dispersal (and gene flow), to ecological and biological traits.

Our results suggest that all individuals in each of the two studied species belong to a single population. A direct visual evaluation of all the presented figures for the STRUCTURE analyses corroborates this statement. Despite the initial raw indication that the populations could be divided into two or three genetic clusters, this was not upheld after careful analysis of the results. For *C. polykomos*, the first clue for this conclusion was the low values of ΔK and the small likelihood differences between successive K values, which indicated that the strength of the signal detected in the STRUCTURE analysis was weak (Evanno *et al*., 2005; Quintela *et al*., 2014). Furthermore, for both species, in the simulations with K=2, each individual exhibited a probability around 50% of belonging to each cluster. The same pattern was found for K=3, with the individuals being assigned to each cluster with around 33% of probability (and results for K=4 and K=5 suggested the same interpretation). Such kind of results is commonly obtained when K=1 is the most probable explanation but the model is forced to run with higher cluster values (Quintela *et al*., 2014). The double approach using Evanno's ΔK method and L(K) was useful in disentangling the results and elucidate the most probable population genetic structure of TNP's red and black-and-white colobus.

There are two main rivers in TNP: the rivers Meno and Hana (Hoppe-Dominik *et al*., 2011). The width of the rivers is markedly seasonal, reaching the maximum in the wet season and the minimum in the dry season. Although both rivers likely represent a barrier for colobine dispersal in most of their extension, there is evidence of colobines crossing rivers in narrow stretches with overlapping canopy from both margins (*P. pennantii*, Chapman and Chapman, 2000; *Nasalis larvatus* Wurmb, 1787, Matsuda *et al*., 2008). Our preliminary results suggest that also for the studied species gene flow occurs across the two main rivers in TNP, but future research with a wider and larger sampling is needed to assess population structure at the scale of the whole TNP. There is a lack of direct evidence in the literature regarding *P. badius* and *C. polykomos*' dispersal limitations, aside from the intrinsic dependence of forest and the restrictions posed by major rivers, such as for *C. polykomos* by the Sassandra River in Ivory Coast (Gonedelé Bi *et al*., 2006, 2012, 2014). There are many examples of primate species whose population genetic structure is influenced by major rivers, from bonobos (Erikson *et al*., 2004), to orangutans (Arora *et al*., 2010) and lemurs (Quéméré *et al*., 2010), among others. Allen *et al*., (2012) sampled six different groups of *P. rufomitratus* at the Kibale National Park, covering almost the whole park. The authors found that all groups were part of a single population living in the park, a result similar to ours. Thus, seemingly, in the absence of continuous habitat barriers in well-preserved forests, colobus monkeys can exhibit high levels of gene flow. Even though social groups are spatially segregated, gene flow within a continuously forested habitat will reduce their genetic differentiation. Recently, Ruiz-Lopez *et al.* (2016) suggested that fire density and the distance to the nearest village are major features that influence red colobus' gene flow across the landscape. Looking at the TNP situation, fires do not represent a threat, in contrast to human density, which is growing continuously and impacting biodiversity, especially in the forest edges of both western and eastern sides (Refisch and Koné, 2005a, 2005b; Covey, 2009; N'Goran *et al*., 2013). By moving away from the surrounding threats, the red and black-and-white colobus may be converging towards the centre of the

park, reducing the distance between groups and exchanging migrants more frequently. Adding to this, social groups often overlap their home ranges and spend much time moving across the forest, especially due to their strict diet (Covey, 2009; Isbell, 2012). In this context, Fleury and Gautier-Hion (1999) even labelled a *Colobus satanas* population in Gabon as "seminomadic" due to the large home ranges and movements displayed by the social groups. Therefore, there are a multitude of factors shaping the population genetic structure of TNP's *P. badius* and *C. polykomos* populations and, as it seems, possibly leading to generally undifferentiated single populations in each species.

As discussed above, colobines display considerable ecological and behavioural plasticity, and regarding TNP's *P. badius* and *C. polykomos* populations there any many such aspects that need to researched to better understand the variables influencing population structure, population dynamics and landscape use. Additionally, a landscape genetics approach can be particularly relevant for primate species in order to answer critical questions, integrate previous ecological and behavioural knowledge, and allow formulation of the most suitable and effective conservation measures (Arroyo-Rodríguez *et al.*, 2012; Arroyo-Rodríguez and Fahrig, 2014). A much larger sample size and a sampling scheme suitable for landscape genetics analyses would be important next steps for future studies of TNP's *P. badius* and *C. polykomos* populations.

5.4 DEMOGRAPHIC HISTORY: VICISSITUDES IN WEST AFRICA

Demographic history inferences for populations and species are highly important to understand past evolutionary history, the impacts of present conditions, and to predict developments given future environmental scenarios. Thus, their relevance ranges from ecological to evolutionary and conservation biology. The detection of demographic fluctuations can be attempted through many methods, from the simpler genetic diversity-based approaches to the complex Bayesian and simulation-based methods. In our study, we used the popular methods implemented in BOTTLENECK (Cornuet and Luikart, 1996; Piry *et al.*, 1999) and MSVAR 1.3 (Storz and Beaumont, 2002), but also the recently developed VAREFF (Nikolic and Chevalet, 2014). The results of our demographic history analyses are summarized below (Table 5.2).

Table 5.2 – Summary of the demographic history analysis significant results, obtained from BOTTLENECK (Cornuet and Luikart, 1996; Piry *et al.*, 1999), MSVAR 1.3 (Storz and Beaumont, 2002) and VAREFF (Nikolic and Chevalet, 2014).

Demographic history					
Species	**Method/Software**				
	BOTTLENECK			**MSVAR 1.3**	**VAREFF**
	IAM	**TPM**	**SMM**		
P. badius	No signal	No signal	Expansion	Stable population	Double bottleneck
C. polykomos	Bottleneck	Bottleneck	No signal	Stable population	Very recent bottleneck

For *P. badius*, the Wilcoxon sign-rank test (Luikart *et al.*, 1998) for heterozygosity excess did not detect a signal of bottleneck, instead supporting a scenario of a stable population in mutation-drift

equilibrium. Nonetheless, under SMM, there was evidence for a population expansion given the significant heterozygosity deficiency observed. MSVAR 1.3, which methods also assume that microsatellites are evolving under SMM, inferred a misleading ancient expansion, that would have started around 1.5 to 2.5 million years ago, this is, in the Early Pleistocene (Lewis and Maslin, 2015). The obtained patterns indicate the absence of any variation between the current and ancestral effective population sizes, an assumption based also in the of the 95% highest posterior density intervals of N0 and N1 and the inconsistent determination of the time window for the occurrence of an hypothetical variation (double peak patterns of mean T). The causes for such stability are not easy to pinpoint from the scant available literature regarding West African environments in the lower Pleistocene. However, the Upper Guinean Forests of West Africa biodiversity hotspot, in which TNP is included, never disappeared completely, surviving to the harsher climatic cycles and serving as refugia for many species (Levinsky *et al*., 2013; Jacquet *et al*., 2015; Nakazawa and Peterson, 2015). This stability might be behind the maintenance of the elevated effective population size over time. It is nevertheless interesting that several colobine species went extinct during the Pliocene-Pleistocene transition (Leakey, 1982; Ting, 2008): a decrease in interspecific competition with extinct colobines could have facilitated *P. badius* to prosper. Some authors even discussed the possible occurrence of a "species turnover pulse" in cercopithecids (Elton, 2007; Frost, 2007), where some species where replaced by others due to climate change.

There are also both technical difficulties inherent to the applied methods of demographic history inference and ecological processes that are able to generate false expansion signals (Girod *et al*., 2011; Leblois *et al*., 2014), such as asymmetrical gene flow (Paz-Vinas *et al*., 2013). The performance of BOTTLENECK and MSVAR 1.3 has been assessed several times (Chikhi *et al*., 2010; Girod *et al*., 2011; Peery *et al*., 2012), highlighting both their positive features and limitations. Concerning expansions, the two programs are expected to be very reliable, especially the latter (Girod *et al*., 2011). There are fewer published studies in which an expansion signal was found in comparison to bottlenecks signals, making thus harder to evaluate the performance of the methods to reliably infer expansions. It would be also interesting to investigate the demographic history of other West African species to see if similar signals of expansion to that found in *P. badius* could be detected.

VAREFF indicated a steady decrease of Ne until the occurrence of a first severe bottleneck between 150,000 and 140,000 years ago (Late Pleistocene), assuming five years of generation time, that halved Ne from ca. 70,000 to 35,000. This variation was supported by the preliminary runs that showed a decrease between the ancient and the intermediate estimations (positive imbalance index). The time window is consistent with a glacial maximum, "megadrougths" and desertification in tropical Africa, with shrinkage and fragmentation of rainforest extension, creating the Upper Guinea refugia (Pokras and Mix, 1985; Nichol, 1999; Plana, 2004; Cohen *et al*., 2007; Levinsky *et al*., 2013). There are some examples of mammals in West Africa, and inclusively at the TNP, whose demographic history and speciation occurred due to Pleistocene's climate-driven rainforest reductions and extensions (Nicolas *et al*., 2008; Nicolas *et al*., 2011; Jacquet *et al*., 2015). Therefore, it is feasible that climate could have played an important role in the first bottleneck detected for *P. badius*, and the Upper Guinean Pleistocene refugia, in which the forests of TNP are included, might have been crucial for the survival of red colobus populations. The second inferred bottleneck occurred much later, in the last 20 generations (i.e. in the last 100 years), after a stationary phase with a median Ne of 28,000 individuals. Abruptly, these numbers were reduced to a maximum Ne of 100. This result was again supported by the positive imbalance indices, but the magnitude of the decline and the estimates of current Ne (3303 – 14252) differed between the preliminary runs, in which the parameters were based on, and the final simulation (HPD 95% = 13.4 – 107.5). We believe that it is very unlikely that *P. badius* has suffered such a decline so

rapidly. Neither BOTTLENECK nor MSVAR 1.3 supported such scenario, which is also at variance with the high genetic diversity we found. While the occurrence of a population size reduction remains possible, essentially due to bushmeat hunting, such an abrupt decline may not correspond to reality. The current population size estimated by MSVAR 1.3 overlaps in magnitude with that for the last stationary phase given by VAREFF: an overall estimate greater than 20,000 breeding individuals at the TNP. However, both programs failed to converge to the same demographic history and did not inferred mutually incompatible demographic events. Integrating all the results, it is possible that *P. badius* could have gone through an ancient expansion, an intermediate bottleneck, and a recent bottleneck. MSVAR 1.3 is reported to have better performances for detecting more ancient events, while VAREFF may be more powerful to identify recent demographic changes. Regarding the main sources of false bottleneck signals in MSVAR 1.3 (population structure, sampling scheme, among others; Chikhi *et al.*, 2010), we believe that none played a major role in our analysis. In the absence of similar empirical and simulation--based reviews of VAREFF, it is more cautious to doubt the reality of the second, more recent, bottleneck signal indicated by this method, without excluding the possibility of a recent population size decline. Therefore, accepting a Ne around 20,000 individuals, and given the observed high genetic diversity, we may consider the TNP's *P. badius* population as likely healthy from a genetic diversity point of view and that still maintains its evolutionary potential, but ongoing human-induced threats may be changing this status at the moment.

For *C. polykomos*, BOTTLENECK (under IAM and TPM) and VAREFF suggested the same scenario, a demographic bottleneck, while MSVAR 1.3 reported a population stability scenario. Again, the explanation behind the results of MSVAR 1.3 lays in interpretation of the reported patterns, as previously mentioned. Biologically, the absence of any demographic variation can be similarly attributed to the role of biodiversity refugia played the Upper Guinea rainforests during climatic variations.

VAREFF estimated an almost invariable Ne of ca. 80,000 until a recent collapse in the last 20 generations, using the more conservative mutation rate. Again, we consider the magnitude of this very recent decline as unrealistic. The hypothesis of decline is of course possible due to the large overexploitation rate that impacts black--and-white colobus at the TNP (Refisch and Koné, 2005a, 2005b), but a reduction to a Ne of less than 50 for the current population seems unlikely. The small number of samples and microsatellites used surely introduced error in our results and, hence, future studies with improved sample size are needed to confirm our conclusion disregarding the inference of VAREFF.

In conclusion, our demographic history analyses suggest a common explanation in the two species for the observed high genetic diversity that characterizes their populations, which is, most likely, the result of a relatively stable and large long-term Ne during recent history. The natural climatic oscillations of the Quaternary played an important role in the fragmentation and loss of habitat and, consequently, in reductions of Ne in *P. badius*, but not at catastrophic levels as observed for many species that suffered recent human-driven demographic and genetic bottlenecks.

5.5 TNP VS CNP: RECENT HABITAT FRAGMENTATION AND HUNTING

After the discussion of the results for the TNP populations (*P. badius* and *C. polykomos*), we compare them with those for CNP's red and black-and-white colobus (*P. temminckii* and *C. polykomos*)

populations, to evaluate the impacts of the two main threats they are currently facing: habitat loss and fragmentation, through deforestation, and hunting. It is clear that the genetic diversity of CNP's colobine populations is much lower (Minhós *et al*., 2013a, 2013b, 2016) than that in the TNP's populations. The comparison with our results and all other studies we summarized in Table 5.1, leaves little doubt about the genetically depauperate status of populations in CNP. On the contrary, quantitatively, the TNP's populations seem to have the healthier genetic status, even if like those in CNP, also suffered a bottleneck. Crucially, although hunting occurs in both parks, habitat fragmentation through deforestation is only present at the CNP, and it is moreover know that deforestation facilitates higher rates of hunting, for bushmeat or for other motivations. Further, the CNP's populations are at the northern limits of both species distributions, while TNP is at the centre, a fact that also has the potential do influence their genetic diversity.

In our study, habitat fragmentation is equivalent to forest discontinuities promoted by deforestation and the consequent transformation of the land in an unsuitable matrix, either by natural events or by human activities. The result is a division of large and continuous forest into isolated fragments that lost many of their original structural attributes. As forest-dependent species, the red and black-and-white colobus will hardly be able to transpose this matrix and move across fragments, thus the matrix represents a strong barrier that significantly limits dispersal. Still, the scenario is not as linear as could be expected due to the colobines' inherent characteristics. Milich *et al*. (2014) studied *P. rufomitratus* groups that live in logged and old-growth areas in Kibale National Park (Uganda). The authors found that the red colobus were capable of accommodating the logging activity due to their behavioural plasticity when there are alternative resources and the habitat is stable, maintaining their densities. However, this may not be possible in forest fragments due to the shortage of habitat quality and variability, and the consequent lack of alternatives and opportunities to adapt. Regarding black-and--white colobus, the effects of forest fragmentation on the density of *C. angolensis* in coastal southern Kenya was assessed by Anderson *et al*. (2007a). Forest area was the most significant variable affecting the species occupancy and density, while fragment isolation had no effect preventing dispersal. Again, behavioural plasticity was the key to explain the results. Colobines do not seem to follow any strict pattern of response to different challenges and threats and being aware of their intra-and interspecific variation can be crucial for the conservation and long-term viability of populations and species. In small fragments, the decreased possibilities of response by plasticity can drastically reduce the chances of colobines persistence.

Hunting primates for bushmeat consumption, and its "evolution" into a commercial activity, is a practice that is widespread across Africa and that makes very difficult the assessment of the impact of habitat fragmentation. Nonetheless, it is clear that with smaller and more accessible forest fragments comes a greater hunting pressure. The detectability of colobines due to their communications and ecology represents a third factor in this equation of threats to their survival. The studies by Rovero *et al*., (2012, 2015), Araldi *et al*., (2014) and Ruiz-Lopez *et al*. (2016) on the populations of *P. gordonorum* and *C. angolensis* in the forests of the Udzungwa Mountains National Park, Tanzania, all found that hunting is playing a major role in the extinction of the colobine populations and that this threat has been fuelled by the restriction of populations to forest fragments in which individuals are easily targeted. The latter study found that isolation in forest fragments is promoting genetic differentiation of populations, but this diversification is likely short-lived as genetic drift and inbreeding will erode genetic variation and eventually lead to extinction.

The comparison between *P. badius* and *C. polykomos* in our study is hampered by the small sample size for the latter species, but our results on the genetic diversity and allelic richness suggest that their differences may not be very substantial. There is evidence that *C. polykomos* is more hunted at the TNP,

with an overexploitation rate of 156.52% against the 80.38% reported for *P. badius* (Refisch and Koné, 2005a, 2005b), contrarily to the hunting patterns described for CNP (Minhós *et al*., 2013a). Despite their greater ecological resilience, in comparison to red colobus, the black-and-white colobus population will certainly collapse if current harvest levels are maintained. There is much to learn about the colobines behavioural plasticity to habitat modifications, but surely they will not be able to cope with this direct and intense threat unless conservation measures are implemented. Hunting can be accepted as a strong reason behind VAREFF's signals of recent bottleneck for both species, even being sceptical about their magnitude. In contrast to the CNP, in the TNP hunting is acting without the synergistic effect of habitat fragmentation. The absence of fragmentation helps sustaining the genetic variability of populations, the ability of individuals to avoid and escape hunters, and is vital for maintaining population numbers. Although the effective size of the populations may still be high, they have been decreasing and the decline is already detectable. In addition to hunting, the *P. badius* population of TNP has a high rate of retroviruses infection that may have been increasing the mortality rate (Leendertz *et al*., 2010).

The CNP harbours Guinea-Bissau's highest population densities of *P. temminckii* and *C. polykomos*, which however contain less than 500 individuals and with reduced genetic variability (Minhós *et al*., 2016). As previously stated, the loss of genetic diversity settles on a game between four stochastic variables, and in populations with a reduced effective population size, genetic drift becomes the MVP. Of course that, in this case, the title lacks any advantage and the eased rate of fixation and loss of alleles leads to a decrease in the evolutionary potential of the populations or species. Therefore, the long-term viability of the populations is compromised and it is essential that population sizes do not decrease even further. At the TNP, the several thousands of individuals that compose both the red and black-and-white populations still maintain one of the highest genetic diversities among all colobine populations that have been studied, and thus the long-term viability is likely if the hunting pressure is removed or greatly decreased. Following Radespiel and Bruford's (2014) concept of fragmentation genetics, in our case the most appropriate interpretation is that the hunting component cannot be neglected.

5.6 CONTRIBUTIONS FOR CONSERVATION

The TNP contains the most extensive, continuous and well-preserved West African forest. In Ivory Coast, all the remaining protected areas are being deforested for agriculture to supply the international markets of palm oil, rubber and cocoa, but also to support the rapid growth of the human population (Bitty *et al*., 2015). There is an important sociological element that cannot be overlooked. Agriculture and hunting represent vital sources of income and food for local populations. As stated above, there are three main motivations for hunting: culture, food and money (Harrison *et al*., 2016). It can be argued that it is impossible, and even immoral, to tell to local communities to stop activities that guarantee their survival because they are jeopardizing wildlife. Explaining the opportunities that may arise from a well-preserved forest and biodiversity is not simple, especially due to the time it may take to effectively bring advantages to local populations, but it is a crucial effort in conservation.

In a recent review, Adams *et al*., (2016) used the term forest landscape restoration to assess the socioeconomic benefits that large-scale reforestation has for local communities. The authors end the article saying that there are many possible opportunities and advantages in forest recovery, but, despite being a promising approach, it has little implementation in Africa and the long-term outcomes are yet

unknown. Although fragmentation does not exist at TNP, the forests that surrounded the park have gone beyond fragmentation and many have disappeared (Brou Yao *et al*., 2005; Chatelain *et al*., 2010). We believe that the most effective way to recover those forests would be implementing projects for community-based management of the forest fragments. This could provide economic benefits to the human local populations, and would also help Taï's colobines through the establishment of corridors connecting the remaining neighbouring forest blocks and prevent the TNP isolation. By increasing dispersal and gene flow, some of the *P. badius* and *C. polykomos* populations around the TNP could be perhaps salvaged from extinction. As forest-dependent animals, *P. badius* and *C. polykomos* can be used as indicator species of local forests status and conservation. Of course, this would require local people engagement and implementation of a better monitoring system to control hunting. But how? We can give three motivations that could be used not only for the TNP, but also for the CNP.

Koné *et al*., (2008) focused on the role that primates play in the maintenance of the tropical forest equilibrium through their mediated seed dispersal. At the TNP, primates are responsible for the majority of the seed dispersal, surpassing even birds. Many of the trees they help to spread have an economic (for medicine or food) and/or cultural value to local human populations. Therefore, the conservation of primates is crucial not only for forest regeneration, but also for human habitat use. Secondly, Amin and Koné (2015) assessed the relationship between people and protected areas according to the trade-off between costs and benefits of conservation in the forest of Marais Tanoé-Ehy (southeastern Ivory Coast), where *P. waldronae* is present and the remaining colobines are endangered. The authors concluded that, in the current situation, the conservation of the forest bears more costs than direct benefits and the local people, even if willing to preserve the area, could not afford it. Further, there was an unequal distribution of the benefits, in which the richer gained most benefits and the poorest suffered most of the costs. The recent development of the socio-ecological economics and ecosystem services concepts can be the key for the correct valuation of the area. For example, when the authors accounted for the tonnes of carbon dioxide emissions sequestered by the forest and the potential for both tourism and scientific research, the income surpassed 60 million of dollars. Regarding the TNP, the "Nature and Culture" ecotourism plan has been implemented in the park and, despite the absence of profit evidence, the project can be found online promoting the attractions of the forest, in which the observation of *P. badius* groups is included. Finally, the transmission of zoonosis (Wolfe *et al*., 2005) and viral infections (Leendertz *et al*., 2010), including the Ebola virus (Osterholm *et al*., 2015), through contact with bushmeat is a reality in the whole of West Africa. The populations must be educated and warned about these issues in order to avert disease outbreaks and endangering human health. This could lead to the reduction of the current hunting pressure and overexploitation and hence contribute to the long-term viability of the TNP's *P. badius* and *C. polykomos* populations. We suggest that the combination of the three approaches could help sustaining and restoring healthy interconnected forests and primate communities in both Ivory Coast and Guinea-Bissau.

5.7 LIMITATIONS OF THE STUDY AND FUTURE PERSPECTIVES

We attempted, as a parallel project to this study, the development of a protocol to amplify carrion fly-derived primate nuclear DNA, an example of environmental DNA (eDNA), but unfortunately we were unsuccessful in establishing it. The aim was to increase the number of samples used in the study, but also to illustrate an approach that could be applied in similar studies in other animal species. Taberlet *et al*., (2012) reviewed the possibilities of eDNA in ecology and they range from biodiversity

assessments of plants and animals in large areas, to diet analysis and biomonitoring. Therefore, employing successfully eDNA collected in field surveys will stimulate more studies based in this methodology and decrease the effort of large-scale sampling surveys. In particular, the use of carnivorous flies to detect the presence of other animals (Calvignac-Spencer *et al*., 2013; Schubert *et al*., 2015) is truly promising.

There were a few limitations in our study that constrained the methodological options that we could apply to the data and that may have affected the results of the analyses. Foremost among those limitations was the sample size, but also the sampling scheme. The sampling scheme can have important impacts in several population genetic analyses and, consequently, in the interpretation of results (Chikhi *et al*., 2010; Hoban *et al*., 2013; Radespiel and Bruford, 2014). A more directed and systematic sampling scheme, with sample georeferencing to allow landscape genetic approaches, would have been useful and should be conducted in future studies building upon this one. The number of samples was one of the most emphasized problems in our study, especially regarding *C. polykomos*. This kind of studies require relatively large sample sizes in order to prevent biases and misinterpretations (Leberg, 2002; Hale *et al*., 2012; Hoban *et al*., 2013) and the seven samples that we had for the *C. polykomos'* population at the TNP were certainly not enough to represent the population and, therefore, the results for it must be seen as preliminary. A possibility, when increasing sample size is difficult or impossible, would be to increase the number of microsatellites analysed (Hoban *et al*., 2013). Nevertheless, given the limited number of population genetics studies on West African colobines, the possibility of analysing the Ivorian samples of *C. polykomos* could not be neglected and they contribute to the relevance of this study.

In our study, moreover, we only used microsatellites as molecular markers and it would be useful to analyze mitochondrial DNA as well. For instance, this could help to clarify the sex-biased dispersal questions that remain. Another avenue would be to jump from population genetics to population genomics, and from conservation genetics to conservation genomics. Allendorf *et al*. (2010) defined conservation genomics as "the use of new genomic techniques to solve problems in conservation biology", following Frankham's definition of conservation genetics (Frankham, 1995). The main differences reside on the molecular markers used and in the "new genomic techniques" that took to a whole new level the amount of collected data. The use of single nucleotide polymorphisms (SNPs) and next generation sequencing (NGS) methodologies have expanded the horizons of the questions that can be answered, like adaptive variation, genotype-environment interactions and the genetic basis of inbreeding depression (Salgado-Lynn *et al*., 2016). There is no doubt of the contributions that conservation genomics can provide to conservation and research on non-model organisms in general (Shafer *et al*., 2015).

6 FINAL REMARKS

The Anthropocene will make us rethink all the strategies that ensure the equilibrium between humans and biodiversity. Many of the threats are not new but it is on our hands whether they increase or decrease. In Africa, a great extent of land that once supported rainforests now supports the growth of agricultural species with direct economic value. The species that thrived in those forests were hunted either for food or trade, or became extinct due to the ecological pressures and stresses. Why? Because the local human populations did not (and many still do not) have other alternatives for their own survival. The self-sufficiency scenario of the past is evolving into commercial exploitation that, in many cases, seeks to respond to international demands. The opportunity of gaining direct and real profit from natural resources is the driving force of biodiversity decline. Creating laws that prohibit these activities will be as efficient as the opportunities that arise in parallel with their implementation. Therefore, it is crucial to enrol the local communities in the conservation of the natural heritage, monitoring and effectively encouraging the creation of a new equilibrium between humans and biodiversity. This is the only path towards the conservation of the colobines in both TNP and CNP, but also everywhere when the same struggle is present.

The genetic assessment of populations provides information across a timeline that goes from the past, passes the present and extends towards future predictions. Knowing what were and what are the main variables influencing a population at a given location helps designing the most effective conservation measures and take into account future change. Even though the TNP's red colobus and black-and-white populations still maintain a high genetic diversity, hunting is reducing their numbers and a recent method for demographic history inference suggested recent bottlenecks. The populations are isolated in the biggest and better preserved extension of rainforest in West Africa, and creating forest corridors to reconnect it with the major surrounding forests would be especially important for the species. However, the situation in the Ivory Coast is not favourable and these measures should not be implemented at the cost of the TNP and its extraordinary biodiversity. Again, a socio-ecologic economic development of the region if of upmost importance, as it is for Guinea-Bissau. In fact, the situation in Cantanhez is much worse and the small local colobine populations need strong protection to ensure their persistence. If nothing is done, Guinea-Bissau's largest populations of *P. temminckii* and *C. polykomos* will vanish rapidly.

Concluding, our study is, to our knowledge, the first to investigate the genetic structure and the demographic history of the populations of *Piliocolobus badius* and *Colobus polykomos* that inhabit the Taï National Park. Our results indicate historical natural variations in the species effective population size, but also recent and ongoing declines that are likely anthropogenic. A literature review of the genetic diversity in colobine populations and species allowed concluding that, on one hand, our study populations seem still rich in term of genetic diversity, despite the heavy hunting pressure, and, on the other hand, the populations of Cantanhez National Park are among the most impoverished genetically. In the future, Taï's colobine populations should be studied with landscape approaches at the scale of the whole park to better understand population dynamics and assess if there are any cryptic barriers inside the park. The behavioural plasticity of the species may provide resilience to some threats and little is known on this regard about the populations we studied. Lastly, a population genomics approach may help to trace important signals of local adaptation and interactions with the environment.

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APPENDICES

1 SAMPLE DETAILS OF *P. BADIUS* AND *C. POLYKOMOS*

AT 1 - Sample details for *P. badius* and *C. polykomos*. NExt = Extraction number; AliqQ = Aliquot quantity in µL.

Piliocolobus badius				
Individual ID (NExt)	**Biological Material**	**Concentration**	**AliqQ (µL)**	**Dilution**
3.6	Liver	489.1	4.1	66.8436667
12.15	Spleen	582.5	3.4	66.0166667
23.5	Liver	476	4.2	66.64
44.2	Intestines	537.73	3.7	66.3200333
68.7	Axilary LN	267	7.5	66.75
71.2	Lung	461.5	4.3	66.1483333
72.3	Liver	1314	1.5	65.7
106.2	Spleen	770	2.6	66.7333333
107.2	Spleen	676	3	67.6
108.2	Spleen	820	2.4	65.6
109.2	Spleen	834	2.4	66.72
110.2	Spleen	289	6.9	66.47
111.2	Spleen	543	3.7	66.97
128.2	Buffy coat	229	8.7	66.41
133.2	Buffy coat	372.3	5.4	67.014
213.2.2	Kidney	291	6.9	66.93
215.1.2	Mesenterial LN	368	5.4	66.24
236.2	Submandibular LN	492	4.1	67.24
254.2	Intestines	215	9.3	66.65
268.4	Submandibular LN	206	9.7	66.6066667
270.1	Intestines	278	7.2	66.72
273.2	Marrow	493	4.1	67.3766667
274.1	Marrow	1704	1.2	68.16
275.1	Heart	422	4.7	66.1133333
276.4	Spleen	1525	1.3	66.0833333
278.3	Meat	258	7.8	67.08
279.2	Marrow	652	3.1	67.3733333
280.1	Meat	233	8.6	66.7933333
439.1	Intestines	269	7.4	66.3533333
470.4	Lymph node	591	3.4	66.98
485.5	Lung	438	4.6	67.16
Colobus polykomos				
Individual ID (NExt)	**Biological Material**	**Concentration**	**AliqQ (µL)**	**Dilution**
134.1.3	Buffy coat	32.1		
136.1.3	Buffy coat	35		
224.1.1	Buffy coat	159		
225.3	Buffy coat	41	20	27.3333333
226.2	Buffy coat	124	16.1	66.5466667
237.2.1	Intestine	550	3.6	66
442.1	Bone marrow	663	3	66.3
740.9	Trachea	359.85	5.6	67.172
769.1	Blood	135	14.8	66.6

2 PRIMERS SEQUENCES

AT 2 - Primers used in the study. Source of the primers sequences: Cooperative Human Linkage Center (CHLC); Fesps – Polymeropoulos *et al*. 1991.

Primer/Locus	Primer Sequence (5'- 3')	
	Forward primer	Reverse primer
D2s1326	F: AGACAGTCAAGAATAACTGCCC	R: ATCTGCTGTGACCCAAAAGC
D4s2408	F: AATAAACTTCAACTTCAATTCATCC	R: AGGTAAAGGCTCTTCTTGGC
D6s474	F: TGTACAAAAGCCTATTTAGTCAGG	R: TCATGTGAGCCAATTCCTCT
D10s611	F: CATACAGGAAACTGTGTAGTGC	R: TTACACAAAATATTTACATTCACTTATG
D13s321	F: TACCAACATGTTCATTGTAGATAGA	R: CATACACCTGTGGACCCATC
D1s548	F: GAACTCATTGGCAAAAGGAA	R: GCCTCTTTGTTGCAGTGATT
D2s442	F: AAGGGAAGGAGCATAGCAAC	R: CACCAATAGGATTAGATAGATTAGACA
D11s2002	F: CATGGCCCTTCTTTTCATAG	R: CCTCCCCCTAATGCTGGTAT
D12s372	F: TGGACCACAGGGTATCATCT	R: AGGGCTTGGGTGAATTGAG
Fesps	F: GGAAGATGGAGTGGCTGTTA	R: CTCCAGCCTGGCGAAAGAAT
D1s1665	F: TAAGTAAGTTCAAATTCATCAGTGC	R: TTCCAAGCTTCACAGTGTCA
D6s503	F: CGGTTCAGTCCATAGCAACT	R: TCCAAACTTTAAAATGTCCTAACAA
D6s1056	F: ACAAGAACAGCATGGGGTAA	R: GCATGGTGGACTATTTGGAT
D10s676	F: GAGAACAGACCCCCAAATCT	R: TGCAATAAAATAGAAAATGTCAGA
D10s1432	F: CAGTGGACACTAAACACAATCC	R: AGCCTGGGTGACAGAGTGAG
DEAD-box	F: TGATGTTTAGTGCTACTTTTCCTAAGGAA	R: AGAGGTAGAGCCWACTCTKCCTACA

3 PRIORS AND HYPERPRIORS FOR MSVAR 1.3 SIMULATIONS

AT 3 - Starting values for priors and hyperpriors used in the MSVAR 1.3 simulations.

	Priors				Hyperpriors			
	log(N0)	log(N1)	logϴ	log(T)	log(N0)	log(N0)	logϴ	log(T)
Scenario 1	4 1	4 1	-3.5 1	5 1	6 2 0 0.5	5 2 0 0.5	-3.5 0.25 0 0.5	5 2 0 0.5
Scenario 2	3 1	5 1	-3.5 1	5 1	4 2 0 0.5	4 2 0 0.5	-3.5 0.25 0 0.5	5 2 0 0.5
Scenario 3	5 1	3 1	-3.5 1	4 1	4 2 0 0.5	5 2 0 0.5	-3.5 0.25 0 0.5	5 2 0 0.5
Scenario 4	5 1	4 1	-3.5 1	4 1	3 2 0 0.5	5 2 0 0.5	-3.5 0.25 0 0.5	5 2 0 0.5
Scenario 5	4 1	5 1	-3.5 1	5 1	5 2 0 0.5	3 2 0 0.5	-3.5 0.25 0 0.5	5 2 0 0.5

4 NULL ALLELE ANALYSIS OUTPUTS

AT 4 - Null allele analysis outputs for *P. badius* and *C. polykomos*. Results from MICROCHECKER

Piliocolobus badius					
Locus	**Null Present**	**Oosterhout**	**Chakraborty**	**Brookfield 1**	**Brookfield 2**
D1s1665	no	-0.0256	-0.0158	-0.0133	0
D4s2408	no	-0.0367	-0.0359	-0.0338	0.0631
D13s321	no	-0.0222	-0.0206	-0.0185	0.1345
D6s474	no	-0.0046	0.0128	0.011	0.011
D2s1326	no	0.0471	0.0395	0.0331	0.119
Fesps	no	0.012	0.0101	0.0087	0.102
D11s2002	no	0.0458	0.0467	0.0405	0.0405
D2s442	yes	0.1267	0.1464	0.1195	0.1195
D6s503	no	-0.0035	-0.0026	-0.0024	0.0809
D6s1056	no	-0.0181	-0.0124	-0.0112	0
D10s676	no	-0.0434	-0.0422	-0.0398	0
Colobus polykomos					
Locus	**Null Present**	**Oosterhout**	**Chakraborty**	**Brookfield 1**	**Brookfield 2**
D1s548	no	0.036	0.04	0.0345	0.0345
D1s1665	no	-0.0384	-0.0435	-0.037	0
D4s2408	no	0.0455	0.0533	0.0415	0.0415
D13s321	no	0.0748	0.1064	0.0667	0.3117
D6s474	no	-0.1089	-0.094	-0.094	0
D2s1326	no	0.1459	0.1795	0.1273	0.1273
Fesps	no	-0.1074	-0.0927	-0.086	0
D11s2002	yes	0.1936	0.2471	0.1795	0.1795
D2s442	no	-0.1149	-0.0987	-0.0987	0
D6s503	no	-0.0145	-0.009	-0.0084	0
D6s1056	no	0.092	0.1049	0.0725	0.0725
D10s676	yes	0.3746	0.6893	0.3302	0.3302

5 STRUCTURE HARVESTER NUMERIC RESULTS

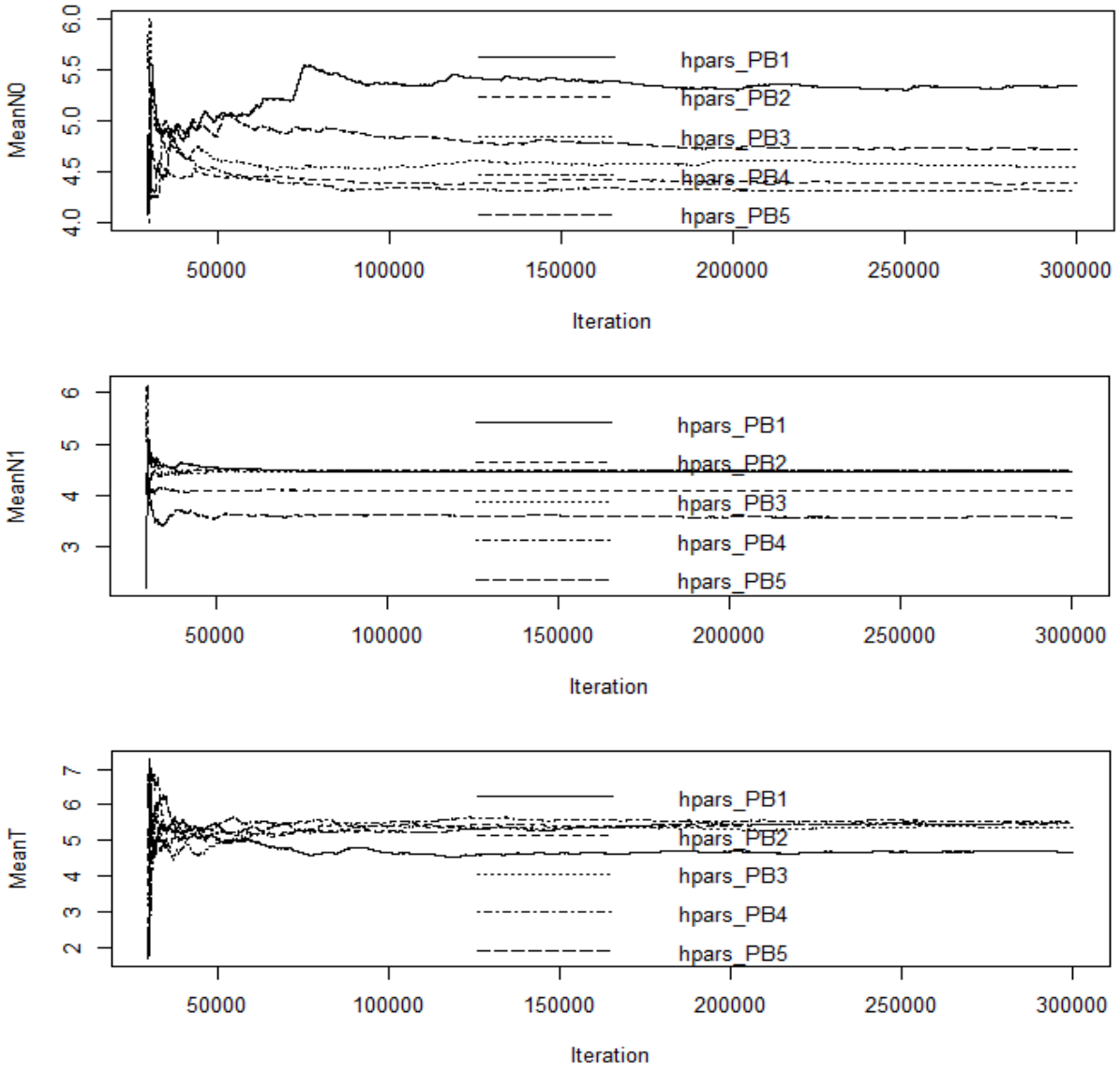
AT 5 - Structure Harvester results from the Structure outcomes for *P. badius*.

Piliocolobus badius						
K	**Reps**	**Mean LnP(K)**	**Stdev LnP(K)**	**Ln'(K)**	**|Ln''(K)|**	**ΔK**
1	5	-1118.160	0.358	—	—	—
2	5	-1138.600	8.213	-20.440	30.960	3.770
3	5	-1190.000	23.229	-51.400	122.700	5.282
4	5	-1364.100	80.386	-174.100	250.400	3.115
5	5	-1788.600	264.912	-424.500	—	—

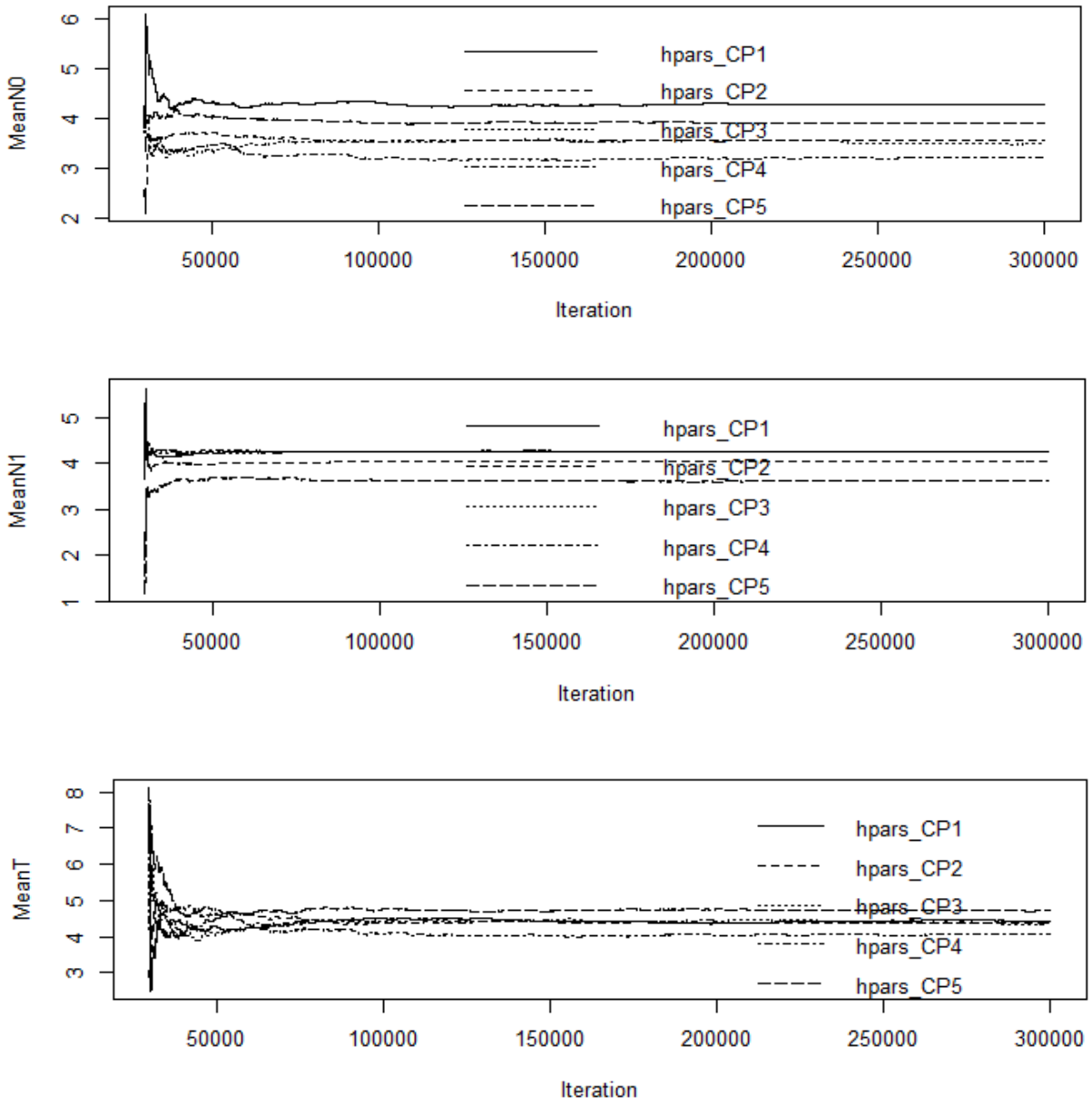
AT 6 - Structure Harvester results from the Structure outcomes for *C. polykomos*.

Colobus polykomos						
K	**Reps**	**Mean LnP(K)**	**Stdev LnP(K)**	**Ln'(K)**	**|Ln''(K)|**	**ΔK**
1	5	-295.0200	0.286	—	—	—
2	5	-294.360	1.396	0.660	2.100	1.504
3	5	-295.800	1.098	-1.440	0.900	0.820
4	5	-296.340	1.078	-0.540	0.440	0.408
5	5	-296.440	1.464	-0.100	—	—

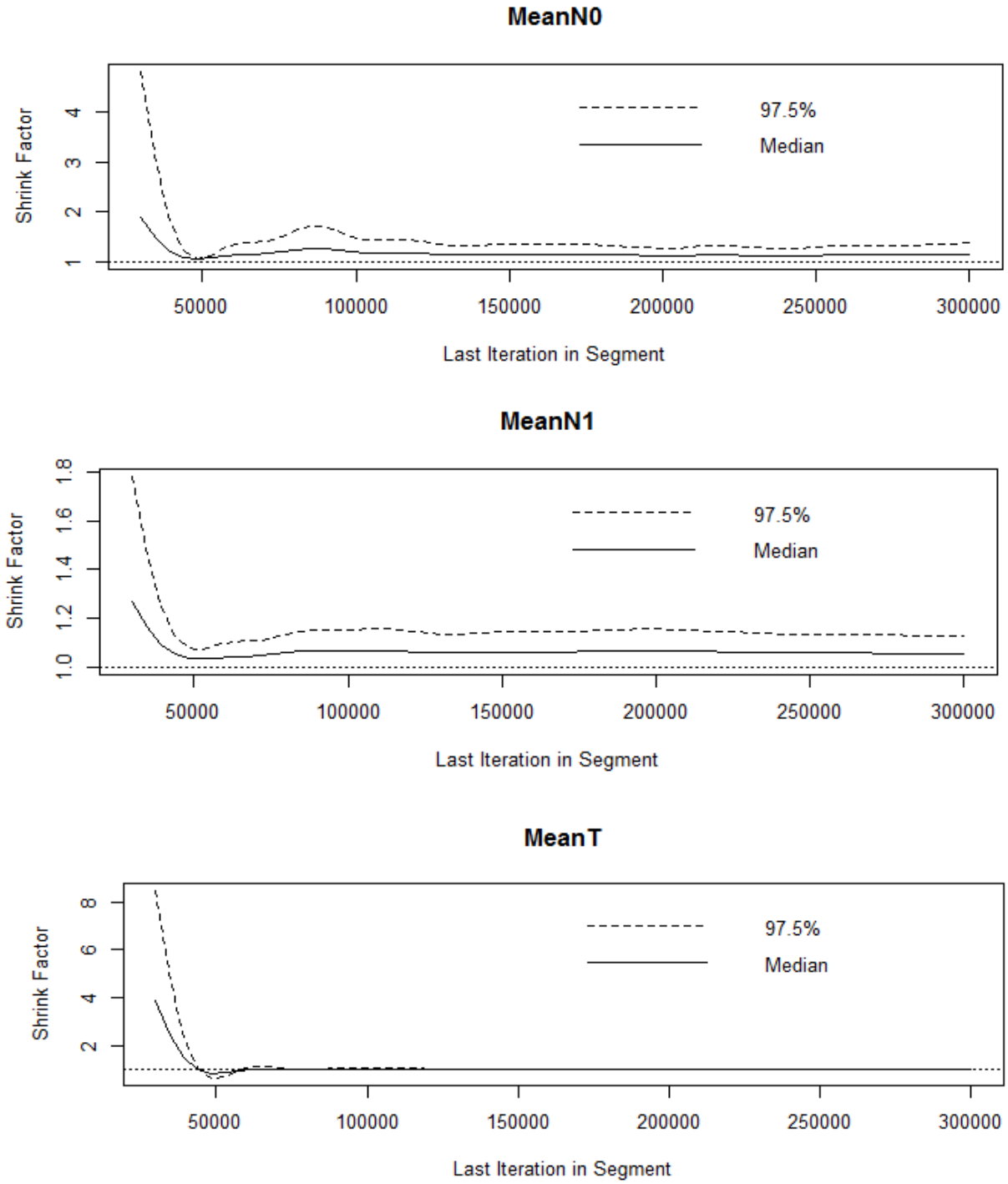
6 DEMOGRAPHIC HISTORY SECONDARY OUTPUTS



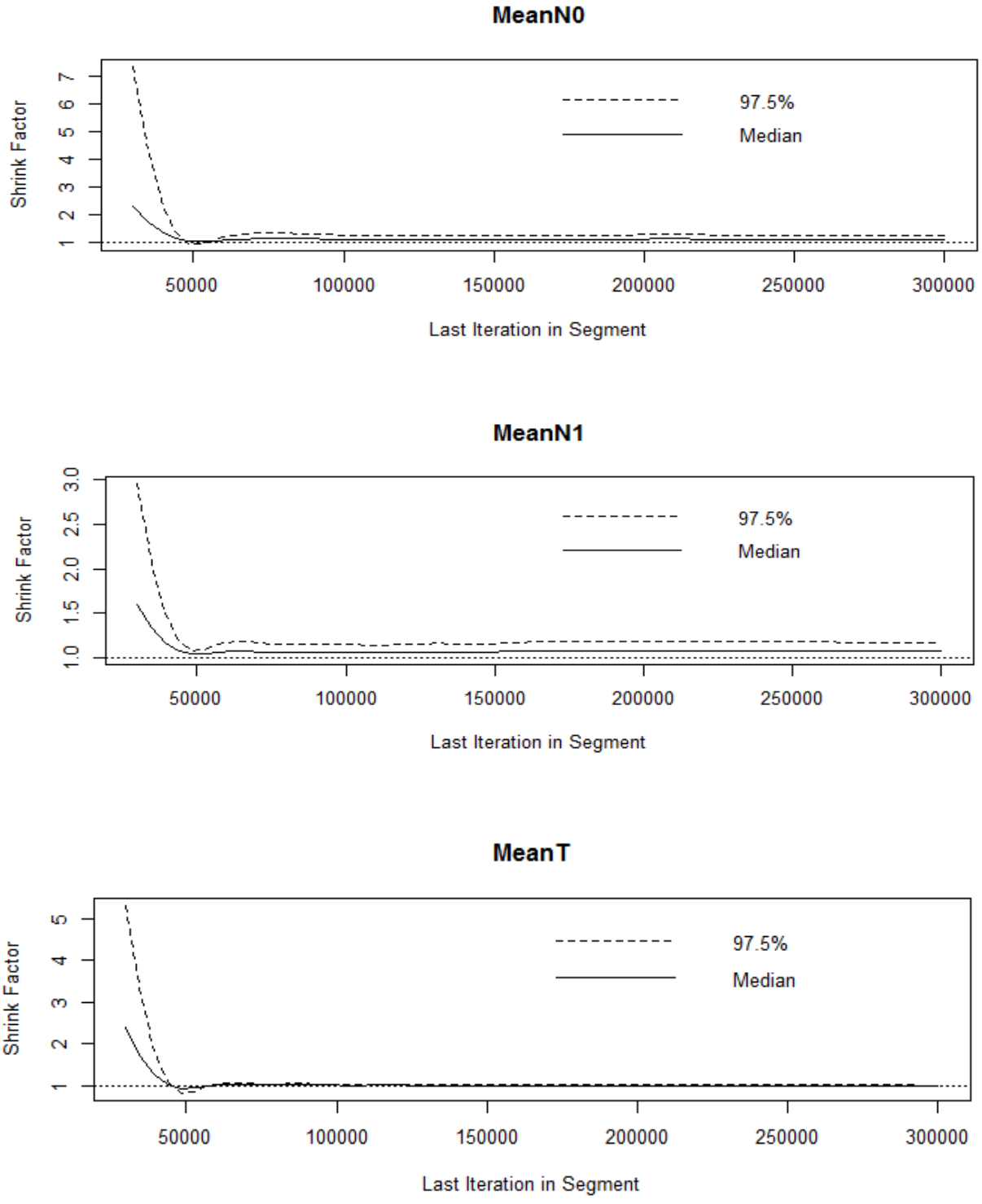
AF 1 – Variation of mean N0 (top), N1 (middle) and T (bottom) across the iterations for *P. badius*. From BOA v. 1.1.7-2.



AF 2 - Variation of mean N0 (top), N1 (middle) and T (bottom) across the iterations for *C.polykomos*. From BOA v. 1.1.7-2.



AF 3 - Brooks, Gelman and Rubin Convergence Diagnostic test for *P. badius*. Variation of the shrink factor of N0 (top), N1 (middle) and T (bottom) across the iterations. From boa v. 1.1.7-2.



AF 4 - Brooks, Gelman and Rubin Convergence Diagnostic test for *C. polykomos*. Variation of the shrink factor of N0 (top), N1 (middle) and T (bottom) across the iterations. From boa v. 1.1.7-2.